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STUDIES ON BIOLUMINESCENCE.

VII. Reversibility of the Photogenic Reaction in Cypridina.

By E. NEWTON HARVEY.

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(Received for publication, September 3, 1918.)

In a previous paper of this series,[1] I have described two photogenic substances in the ostracod crustacean, *Cypridina hilgendorfii*, which I called photogenin and photophelein. Photogenin is destroyed below the boiling point, is non-dialyzable, and prepared by making a cold water extract of the luminous animal and allowing it to stand in the air until no more light appears on shaking. This indicates that one of the photogenic substances, photophelein, has disappeared, leaving the photogenin. Photophelein is not destroyed by short boiling and will dialyze. It is prepared by making a hot water extract of the luminous animal. The hot water destroys the photogenin, leaving the photophelein. Whenever two such non-luminous solutions are mixed light appears.

On the grounds of method of preparation, relation to temperature, and dialysis, I regarded photogenin as comparable to luciferase and photophelein as comparable to luciferin, two photogenic substances described by Dubois[2] in the beetle, *Pyrophorus noctilucans*, and in the mollusc, *Pholas dactylus*. Dubois believes that luciferase is an oxidizing enzyme which oxidizes luciferin, an oxidizable substance, with light production. Neither luciferase nor luciferin alone in solution can produce light but light appears if solutions of the two are mixed and continues as long as any luciferin remains unoxidized. Dubois has also been able to produce light by oxidizing luciferin (alone) with a small crystal of $KMnO_4$, by H_2O_2 (with or without blood containing

[1] Harvey, E. N., *Am. J. Physiol.*, 1917, xlii, 318.

[2] Dubois, R., *Compt. rend. Soc. biol.*, 1885, xxxvii, 559.

hemoglobin), BaO_2, PbO_2, and other oxidizing agents. Through the kindness of Professor Dubois I have received some material of *Pholas dactylus* preserved in sugar and I can confirm his results on the effect of $KMnO_4$ and other oxidizing agents in producing light with luciferin of *Pholas*. I have likewise repeated my own experiments with the photophelein of *Cypridina* using a whole series of oxidizing agents applied in the same way, as with the luciferin of *Pholas* and, as previously, have failed to obtain any light with this substance.[3] The difference in our results is, therefore, not to be referred to a difference in method of experiment but to a difference in the substances themselves.

I found also that if one takes a concentrated solution of photogenin filtered through a porcelain or silicious filter candle to remove all granules and cell fragments and adds to it a little saponin powder, or amyl alcohol or NaCl or other inorganic salt crystals or tissue extracts of certain invertebrate non-luminous animals, that light would appear. Because NaCl could not possibly be oxidized by photogenin (= luciferase)—or any other substance—and because of my inability to make photophelein (= luciferin) luminesce with oxidizing agents, I regarded the photogenin itself as the source of the light and the oxidizable body. I have compared photogenin to zymase and photophelein to the coenzyme of zymase, believing that we are dealing with a system similar to that of the enzyme-coenzyme system of yeast. Hence the name photophelein or body assisting in the production of light.

[3] The following oxidizing agents (added ,where possible, in minute crystal or powder form) all gave light with *Pholas* luciferin, but no light with *Cypridina* luciferin: $KMnO_4$, $K_2Cr_2O_7$, PbO_2, Na_2O_2, BaO_2, MnO_2, $K_3Fe(CN)_6$, $K_2S_2O_8$, $Na_2B_4O_8$, and H_2O_2. The following oxidizing agents gave no light with either *Pholas* luciferin or *Cypridina* luciferin: K_2CrO_4, CrO_3, $KClO_3$, $KClO_4$, $FeCl_3$, KNO_3, Cl or Br water, I in KI, Na hypochlorite, hypobromite, or hypoiodite, colloidal Ag or Pt, benzoyl peroxide, potato or turnip juice, or blood containing hemoglobin or hemocyanin. If H_2O_2 in addition to the oxidizing agent is added to *Cypridina* luciferin, no light appears except a faint momentary flash with Na hypochlorite and hypobromite. As this faint flash also appears with thoroughly boiled extracts of *Cypridina*, lacking luciferin, it can have no significance. If H_2O_2 in addition to the oxidizing agent is added to *Pholas* luciferin the light is in some cases brighter than with H_2O_2 alone.

I now believe that under the term photophelein I have previously included two separate substances. One of these is the thermostabile dialyzing substance extracted from *Cypridina* by hot water. Although this substance cannot be oxidized with light production by oxidizing agents, it does oxidize spontaneously (also without light production) in the air and loses its power of producing light with photogenin. In the absence of air its solutions are stabile for months. Once oxidized it can again be reduced and will again give light if photogenin is added. It is therefore an oxidizable material and, I believe, similar to the luciferin of *Pholas*. I propose therefore to use Dubois' word luciferin for the thermostabile dialyzing substance of *Cypridina* in place of photophelein and luciferase for the thermolabile non-dialyzing substance in place of photogenin. The source of the photogenic substances can be designated by prefixing the name of the animal as *Cypridina* luciferin, *Pholas* luciferin, etc. I suggest also that luciferin when oxidized be designated oxyluciferin.

Luciferin is found only in luminous animals. In non-luminous animals and probably also in luminous animals there is a second substance which I have formerly included in the term photophelein, and which may be properly so called, that acts in a manner similar to saponin, NaCl crystals, etc., upon the extract of *Cypridina* which has stood until the light disappears. When we allow a *Cypridina* extract containing luciferin and luciferase to stand, the luciferin is not completely oxidized, even though the extract is thoroughly aerated, but some of it is bound (adsorbed or combined?) by other substances in the extract. The saponin, NaCl crystals, and extracts of non-luminous animals act by setting free the bound luciferin which is then oxidized and light appears. I suggest that the term photophelein be applied to these substances in tissue extracts. They are not destroyed by boiling. On standing some are stable while others are unstable.

The best way to rid a luciferase solution of the bound luciferin is to shake it thoroughly with chloroform. Such a solution will give no light with extracts of non-luminous animals or saponin, NaCl crystals, etc., but a brilliant light with *Cypridina* luciferin.

An insight into the *modus operandi* of saponin, NaCl crystals, or photophelein may be gained from the following experiments. Both luciferin and luciferase are adsorbed by many finely divided precipi-

tates and colloidal particles, such as bone-black, $Fe(OH)_3$, kaolin, and others. If we take a colloidal $Fe(OH)_3$ solution of the proper concentration (which can only be determined by experiment), add some dilute luciferase to it, and then after a minute luciferin, no light will appear. This is because the luciferase has been completely adsorbed by the colloidal $Fe(OH)_3$, for if we now add some dilute luciferase to the above mixture light will appear but it will very quickly disappear because the new luciferase added is again very rapidly adsorbed, but not so rapidly adsorbed that we fail to get light at first. On adding more luciferase we may again get a momentary light but the additions cannot be made indefinitely because we finally reach a point where the colloidal $Fe(OH)_3$ has become saturated with luciferase and then the mixture glows for a considerable time. It is obvious that for this experiment to succeed there must be more luciferin present than can be completely adsorbed by $Fe(OH)_3$ and so little luciferase present that it is completely adsorbed by the $Fe(OH)_3$. Suppose we have a mixture of $Fe(OH)_3$, luciferase, and luciferin complying with the above conditions. Can we in any way remove the luciferase from its adsorbed condition on the colloidal $Fe(OH)_3$? This might theoretically be done in two ways and we actually find in practice that both methods are possible. Anything which precipitates the colloidal $Fe(OH)_3$ will decrease the surface available for adsorption of luciferase and if the surface area is sufficiently decreased some luciferase may be forced into solution again where it is able to oxidize the luciferin. If we add NaCl crystals to the colloidal $Fe(OH)_3$ + dilute luciferase + more concentrated luciferin mixture, the $Fe(OH)_3$ is precipitated and light appears. If in place of NaCl crystals we add a trace of saponin, the colloidal $Fe(OH)_3$ is not precipitated but light also appears. This is an example of the second method of removing luciferase from an adsorbed condition; namely, by using a material (saponin) which is more strongly adsorbed than the luciferase and which is able to replace it as adsorbed body. I believe these to be the explanations of the effect of NaCl crystals, saponin, etc., in giving light with luciferase solutions, except that the luciferase is in excess and a small amount of adsorbed (or combined) luciferin is present which is liberated by NaCl or saponin and gives light with luciferase. Photophelein probably acts in a manner analogous to the saponin.

I have considered the thermostabile, dialyzing substance as similar to the luciferin of *Pholas* despite the fact that Dubois reports *Pholas* luciferin destroyed at 70°C., whereas *Cypridina* luciferin is only destroyed by several minutes boiling in an open beaker. I find that this destruction of *Cypridina* luciferin on short boiling is due to the increased rate of oxidation at the boiling point and that no destruction of *Cypridina* luciferin will occur if boiled in an atmosphere of hydrogen.[4] *Cypridina* luciferin is truly thermostabile but is oxidized to oxyluciferin on boiling in the air. We may say that *Pholas* luciferin is *similar* but certainly not *identical* with *Cypridina* luciferin. If so, we should expect to obtain light on mixing *Pholas* luciferin and *Cypridina* luciferase, yet no light appears. Neither is there light on mixing *Cypridina* luciferin and *Pholas* luciferase, although the *Pholas* luciferase prepared from the material which Dubois sent me gave a rather faint light with *Pholas* luciferin.[5]

We have, therefore, at least three substances concerned in light production: luciferin, luciferase, and photophelein. Luciferin is a body oxidizing with light production, dialyzable, and relatively resistant to heat. Luciferase is destroyed by boiling, non-dialyzable, and accelerates the oxidation of luciferin. While it may be used up in the reaction if mixed with a sufficient quantity of luciferin, luciferase has

[4] I have endeavored to repeat this experiment with the luciferin of *Pholas* sent me by Professor Dubois, but without success. *Pholas* luciferin boiled in a current of hydrogen for 15 minutes would give no light when a crystal of $KMnO_4$ was added. The hydrogen was produced in a Kipp generator and may have contained a little air. In my experience short (20 to 40 seconds) boiling of *Pholas* luciferin does not completely destroy its power of producing light when a crystal of $KMnO_4$ is added.

[5] I believe the faint light obtained on mixing *Cypridina* luciferin and fire-fly or *Noctiluca* luciferase and *vice versa*, recorded in my former paper (*Am. J. Physiol.*, 1917, xlii, 328) where luciferin is called photophelein and luciferase is called photogenin, is not due to the oxidation of luciferin by luciferase of the second species but is due to the presence of photophelein. I am led to this conclusion because the light is so faint, but cannot be sure until the cases are reinvestigated. The mixing of luciferin and luciferase of different species or genera of luminous ostracods, especially if the colors of their luminescence differed, would shed considerable light on this interesting question of specificity. A non-luminous Japanese species of *Cypridina* does not contain either luciferin or luciferase but it does contain photophelein.

many of the characteristics of an enzyme and certainly as much right to be called an enzyme as the peroxidases of plants which are also used up in the oxidation process. The *Cypridina* luciferase reaction appears to be specific to an extraordinary degree. Of many tried I have found no substances or plant or animal extracts which can take the place of luciferase[6] nor any substances[7] or plant or animal extracts[8] which can be oxidized with light production by luciferase. The light recorded with various extracts of luminous and non-luminous animals in my former paper is to be referred to the presence of photophelein, the third substance concerned in light production which probably acts by assisting the luciferin-luciferase reaction in the manner already suggested. Let us now turn to the oxidation product or products of luciferin.

When luciferin is oxidized it must be converted into some substance or substances and I believe this change involves no fundamental destruction of the luciferin molecule, as it is a reversible process. I

[6] I have tried the blood or extracts of many species of animals or plants including those containing strong oxidizing enzymes both with and without H_2O_2 and have always failed to obtain light with *Cypridina* luciferin. Among others the juice of Indian pipe (*Monotropa*), potatoes, and turnips (containing strong oxidases and peroxidases), the blood of the ox and a worm (*Arenicola*) (containing hemoglobin), the blood of the squid (*Loligo*), *Limulus* and *Sycotopus* (containing hemocyanin), and extracts of *Chætopterus* (a luminous annelid), and the mollusc, *Unio*, (rich in manganese) were tried. Dubois reports that he has obtained light on mixing *Pholas* luciferin with the blood of divers molluscs and marine crustaceans (*Ann. Soc. Linn. Lyons*, 1913, **xl**). I can confirm this statement for an extract of *Unio*, but obtained no light with *Limulus* blood, *Sycotopus* blood, squid (*Loligo*) blood, or turnip or potato juice and *Pholas* luciferin. Evidently *Pholas* luciferin is much more readily oxidized with light production than *Cypridina* luciferin.

[7] The following oxidizable substances have been tested: æsculin, lophin, bergamot oil, pyrogallol, gallic acid, aniline, adrenalin, phenol, α-napthol, *p*-phenylenediamine, ortol, orcinol, hydrochinone, resorcinol, pyrocatechol, tannin, benzidine, gum guaiac, amidol, α-napthylamine, and the chromogen of the false indigo plant (*Baptisia*). Luciferase, with or without H_2O_2, will not accelerate the oxidative color change in any of the above compounds.

[8] I have regularly obtained a fair light on mixing luciferase well shaken with chloroform to set free any bound luciferin and boiled potato or turnip juice or boiled *Limulus* blood. The light is especially marked about the coagulum in the boiled *Limulus* blood. The significance of these results is not apparent.

shall speak of the principal if not the only product formed as oxyluciferin. Most observers have considered a rather fundamental change to occur when the photogenic substance is oxidized. Thus the crystals of xanthine or some related substance in the reflecting layer of the fire-fly have been regarded as the oxidation products of the luminous material thought to be nucleoprotein. Dubois[9] regards luciferin as a protein and states that it forms the same oxidation products as other proteins, amino-acids being mentioned as possible substances formed. It should be pointed out in this connection that the formation of amino-acids from proteins involves no oxidation but an hydrolysis.

If we assume that the oxidation of luciferin changes the molecule but slightly, we at once think of comparing the change luciferin $\rightleftarrows$ oxyluciferin with the change reduced hemoglobin $\leftrightarrows$ oxyhemoglobin. The condition is, however, not so simple as this, for oxyhemoglobin will again give up its oxygen providing the partial pressure of oxygen is sufficiently low, whereas oxyluciferin will not do this. We cannot reduce oxyluciferin solution by exhausting the oxygen with an air pump.

There is another oxidation reduction system which can also be easily reversed, but not by merely removing the oxygen—that is the reduction of a dye such as methylene blue to its leuco base. I believe the change which occurs when luciferin is oxidized is similar to that which occurs when the leuco base of methylene blue or sodium indigosulfonate is oxidized to the blue dye.

My attempts to reduce the oxidation product of luciferin started from the observation that if one places a clear solution of luciferase in a tall test-tube, although it may give off no light at first when shaken, after standing a day or so a very bright light would appear on shaking. This was especially true when the luciferase had become turbid and ill smelling from the growth of bacteria. Thinking that the bacteria produced a substance which could be oxidized by the luciferase, I tried growing bacteria and also yeast on appropriate culture media and after some days of growth mixing the culture media containing the products of bacterial or yeast growth with luciferase,

[9] Dubois, *Ann. Soc. Linn. Lyons*, 1914, lxi, 169.

expecting to obtain light. But no light appeared. However, if a little crude luciferase solution was added to the bacterial or yeast cultures and then allowed to stand for some hours, light appeared whenever they were shaken. Indeed such cultures behaved much as a suspension of luminous bacteria which has used up all the oxygen in the culture fluid and will only luminesce when, by shaking, more oxygen dissolves in the culture medium. Realizing that in bacterial cultures in test-tubes anaerobic conditions soon appear, and also the strong reducing action of bacteria upon many substances (for instance nitrates or methylene blue) under anaerobic conditions, it occurred to me that the bacteria might be utilizing the oxygen of the oxidation product of luciferin, reducing it to luciferin again. We must remember that since crude luciferase solution is a cold water extract of a luminous animal allowed to stand until all the luciferin has been oxidized, it must contain oxyluciferin as well as luciferase and will give light if the oxyluciferin is again reduced and oxygen admitted. This appears to be the correct explanation of the above experiments.

Not only bacteria but also tissue extracts have a strong reducing action in absence of oxygen. Thus, muscle tissue stained in methylene blue will very quickly decolorize (reduce) the methylene blue if oxygen (air) is kept away, but the blue color immediately returns if air is admitted. Oxyluciferin, *i.e.* a solution of luciferin which has been completely oxidized by boiling or standing in air until it no longer gives light with luciferase, if mixed with a suspension of ground frog muscle and kept in a well filled and stoppered test-tube for some hours, is reduced to luciferin and gives a bright light if now poured into luciferase solution. Frog muscle suspension alone or oxyluciferin alone gives no light with luciferase, nor will a mixture of frog muscle suspension and oxyluciferin, if shaken with air for several hours. Only if this last mixture is kept under anaerobic conditions is the oxyluciferin reduced.

The reducing action of tissues is said to be due to a reducing enzyme (reducase or reductase), itself composed of a perhydridase and some easily oxidized body such as an aldehyde.[10] In the presence of the

[10] Bach, A., *Biochem. Z.*, 1911, xxxi, 443; xxxiii, 282; 1912, xxxviii, 154; 1913, lii, 412.

perhydridase the oxygen of water oxidizes the aldehyde and the hydrogen set free reduces any easily reducible substance which may be present. There is a perhydridase in fresh milk, spoken of as Schardinger's enzyme,[11] which is destroyed by boiling. If some aldehyde is added, fresh milk will reduce methylene blue to its leuco base or nitrates to nitrites, upon standing a short time. If shaken with air, the blue color returns. There is no reduction unless an aldehyde is added or unless some boiled extract of a tissue such as liver is added. The boiled liver extract has no reducing action of its own but supplies a substance similar to the aldehyde which has been spoken of as a coenzyme. Milk will reduce methylene blue without aldehyde if bacteria are present in large numbers. There is also no reduction if the milk, methylene blue, and aldehyde are agitated with air. The temperature optimum is rather high, 60–70°C.

I find that milk is a favorable and convenient medium for the reduction of oxyluciferin and that it acts without the addition of an aldehyde or the presence of bacteria. There is probably a substance acting as the aldehyde in the luciferase-oxyluciferin solution. No light appears if milk is added to a luciferase-oxyluciferin solution, but if the mixture is allowed to stand in absence of oxygen, light will appear when air is admitted. The air can be conveniently kept out by filling small test-tubes completely with the solution and closing them with rubber stoppers.

Oxyluciferin may also be readily reduced by the use of the blood of the horseshoe crab (*Limulus*) allowed to stand until bacteria develop.[12] This experiment is of special interest because the blood contains hemocyanin which is colorless in the reduced condition and blue in the oxycondition. The color change thus serves as an indicator of the oxygen concentration in the blood. A sample of foul smelling *Limulus* blood full of bacteria will become colorless on standing in a test-tube for 10 or 15 minutes but the blue color quickly returns if shaken with air. Such a blood has the power of reducing oxyluciferin through the activity of the bacteria which it contains. Fresh blood has very little if any reducing action.

[11] Schardinger, F., *Chem. Ztg.*, 1904, xxviii, 704.
[12] Alsberg, C. L., *J. Biol. Chem.*, 1915, xxiii, 495.

As almost all animal tissues contain reductases, it is not surprising to find that a freshly prepared and filtered extract of *Cypridina* containing oxyluciferin and luciferase which gives no light on shaking, will, on standing in a stoppered tube for 24 hours at room temperature, give light when air is admitted.[13] While this may be due to the development of bacteria with a reducing action it does not seem likely, as under the same conditions methylene blue is not reduced in 24 hours and there is no turbidity or smell of decomposition in the tube. In 48 hours bacteria do appear and methylene blue is also reduced. If we add chloroform, toluene, or thymol to the tubes of *Cypridina* extract to prevent the growth of bacteria, and allow them to stand 48 hours, upon admitting air the tube with chloroform gives no light but the tubes with toluene and thymol do give light although it is not so bright as if they were absent. I believe that these substances have a destructive action on the reductases, most complete in the case of chloroform.

I have not been able to demonstrate that a *Cypridina* extract will reduce methylene blue or nitrates to nitrites either with or without the addition of acetaldehyde. This may be due to the fact that oxyluciferin, which is also present, may be reduced more readily than either nitrates or methylene blue and so is reduced first.

Dubois[14] has described in *Pholas* a precursor of luciferin, which he calls proluciferin, which is converted into luciferin by another enzyme, coluciferase. The proluciferin is not destroyed by boiling and the coluciferase will withstand a higher temperature than luciferase and may be freed of luciferase in this manner. He cites an experiment[15] to prove the existence of proluciferin and coluciferase in *Pholas* but I have been unable to repeat this with *Cypridina*. One might suppose that on allowing an extract of *Cypridina* (luciferase) to stand in absence of oxygen, some proluciferin, assuming this to be present, would be converted into luciferin which would give light if air was admitted. But we can allow a boiled extract of *Cypridina* (containing no coluciferase) to stand with milk or muscle tissue suspensions

[13] This experiment may also be performed with *Pholas* luciferase with a similar result.

[14] Dubois, *Compt. rend. Soc. biol.*, 1907, lxii, 850; 1917, lxxx, 964.

[15] Dubois, *Compt. rend. Soc. biol.*, 1917, lxxx, 964.

in absence of oxygen and, upon admitting air and adding luciferase, obtain light. As luciferase is only found in luminous animals it does not seem likely that a coluciferase would be widespread but we do know that a reducing enzyme occurs in milk and tissue extracts—is, in fact, widespread. It seems more logical to interpret the above experiments as due to the reduction of an oxyluciferin to luciferin rather than the conversion of a proluciferin to luciferin.

Indeed, we can reduce oxyluciferin by means which do not involve the use of animal extracts and consequently are free from the objection that "coluciferase" may be responsible for the result, but which, nevertheless, are perfectly well known reducing methods. Perhaps the best of these is reduction by palladium black and sodium hypophosphite. The latter is oxidized in presence of palladium and nascent hydrogen is set free.[16] The nascent hydrogen reduces any easily reducible substance which may be present, such as methylene blue or oxyluciferin. Oxyluciferin is not reduced by palladium alone or hypophosphite alone but methylene blue is reduced by palladium black alone.

If hydrogen sulfide is passed through a solution of methylene blue the dye is very quickly reduced and becomes colorless. If the H_2S is driven off by boiling the colorless methylene blue solution, the blue color again returns on cooling. Oxyluciferin can also be reduced to a certain extent by H_2S. Sulfur dioxide or oxides of nitrogen (prepared by the action of HNO_3 on Cu) had no reducing action on either methylene blue or oxyluciferin.

Dilute acid favors the reduction of oxyluciferin. If one saturates an oxyluciferin solution with CO_2 or adds a little dilute acetic acid and allows the solution to stand for 24 hours, a certain amount of reduction will occur. No reduction occurs if the solution is saturated with pure hydrogen and allowed to stand 24 hours. If one adds some Mg powder to oxyluciferin and then dilute acetic acid in successive additions as the acetic acid is used up in formation of Mg acetate, the oxyluciferin will be reduced relatively quickly. Nascent hydrogen is produced in the reaction and is no doubt the active reducing agent, while the acid accelerates the reduction. Soured milk also has

[16] Bach, *Ber. chem. Ges.*, 1909, xlii, 4463.

quite a marked reducing action. Acid thus favors reduction and hinders oxidation, while alkali favors oxidation and hinders reduction of the oxyluciferin.

While I have not studied the properties of oxyluciferin so fully as those of luciferin, as far as I can judge both substances give the same general reactions and possess identical properties. If we make a concentrated hot water extract of *Cypridina*, it will contain all the substances of the animal soluble in hot water and not coagulated by heat and may be spoken of as crude luciferin solution. If air is bubbled through this solution for some time, all the luciferin is oxidized and it may then be spoken of as crude oxyluciferin solution. Both crude luciferin and crude oxyluciferin solution are yellow in color, but I do not believe that either luciferin or oxyluciferin is yellow in color because an ether or benzene extract of *Cypridina* is also yellow although luciferase, luciferin, and oxyluciferin are all insoluble in ether and benzene. The yellow pigment which can be observed to make up part of the luminous gland of *Cypridina* is not luciferin or luciferase. It may be a pigment related to urochrome.

When tests are applied and precipitating reagents are added to crude luciferin and crude oxyluciferin solution they give identical results in each case. Thus if crude luciferin is saturated with $(NH_4)_2SO_4$ or $MgSO_4$, a flocculent precipitate forms which may be demonstrated to contain most of the luciferin. Oxyluciferin solution also gives flocculent precipitates on saturation with $(NH_4)_2SO_4$ and $MgSO_4$ and these contain most of the oxyluciferin. To demonstrate this the precipitates, after washing, are dissolved in a small amount of water mixed with fresh milk (or frog muscle suspension) and allowed to stand in a stoppered tube for 24 hours. If any oxyluciferin is present it will be reduced to luciferin and give light when luciferase is added. One-half saturation with $(NH_4)_2SO_4$ or $MgSO_4$ or saturation with NaCl salts out no material from either crude luciferin or oxyluciferin solution. Picric acid gives no precipitate but only an opalescence in both cases. In a similar manner it may be shown that most of the oxyluciferin is precipitated by phosphotungstic acid but not by acetic acid or CO_2, in this respect also agreeing with the behavior of luciferin. Like luciferin the oxyluciferin will pass porcelain filters, dialyze through parchment or collodion membranes, is soluble

in absolute alcohol but not in ether or benzene, and is undigested by salivary diastase, pepsin HCl, Merck's pancreatin in neutral solution, and erepsin. The salivary diastase and the pancreatin (containing amylopsin, trypsin, and lipase) were allowed to digest for 4 days at 38°C. without showing any evidence of digestive action. Oxyluciferin is partially but not completely precipitated by basic lead acetate and tannic acid.

As luciferin is so easily oxidizable a substance, we should expect to find that it will reduce just as glucose will reduce. However, a concentrated solution of luciferin has no reducing action on Fehling's (alkaline Cu), Barfoed's (acid Cu), Nylander's (alkaline Bi), or Knapp's (alkaline Hg) reagent. Glucose will reduce methylene blue in alkaline (not in neutral) solution but luciferin will not reduce methylene blue in alkaline or neutral solution. It would seem, then, that luciferin must contain no aldehyde group. If so, we should expect to obtain reduction of some of the above reagents. Just what group is concerned in the oxidation is unknown at the present time and speculation regarding it in the absence of more experimental data can be of little value.

THE PHOTIC SENSITIVITY OF CIONA INTESTINALIS.

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I. Preliminary Statement.

The sensitivity of *Ciona intestinalis* to light furnishes an unusual opportunity for analysis of the receptor process occurring in the sense organ. This is because a sudden increase in illumination produces a vigorous and clear cut response on the part of the animal. Such a reaction to a source of stimulation, which in itself may be measured accurately and controlled with comparative ease, makes the sensory behavior of *Ciona* amenable to a strict, quantitative treatment. My experiments were therefore concerned, first, with the localization of the photic sense organs, and second, with variations in the magnitude of the stimulating agent under conditions calculated to throw light on the nature of the sensory process.

The species used is identical with the *Ciona intestinalis* common in the Mediterranean Sea and along the European coast (Ritter and Forsyth, 1917). At San Diego, California, it is to be found in enormous numbers attached to piles, and forming thick carpets on the under side of floats. In fact, the hundreds of individuals employed were all taken from a single float in San Diego Bay.[1]

II. Photic Sense Organs.

1. *Ciona* possesses two qualitatively different means of reacting to light. In one case the response is a general one, involving the organism as a whole. In the other the activity is confined to the lo-

[1] My thanks are due to the Scripps Institution for enabling me to come to the station at La Jolla, and to the director, Dr. Wm. E. Ritter, and his staff for the many courtesies extended to me during my stay.

cality which receives the stimulation. I shall describe the latter first because of its comparative insignificance in the experiments to be presented.

The body of *Ciona* may be roughly divided into three parts: an anterior region occupied by the siphons and extending below the intersiphonal neural mass; a posterior region containing the viscera and reproductive organs; and a middle region composed in the main, of the translucent branchial sac. The posterior region is practically insensitive to light. The anterior region, *with the notable exception of the area around the neural mass*, is similarly insensitive. The middle portion, however, is quite responsive to photic stimulation.

Light focussed on different parts of this area resulted in a purely local contraction of the circular and longitudinal muscle fibers. When a part of this area on one side was exposed to light, the animal curved on its long axis toward the exposed side. By repeatedly exposing first one side and then the other to this local stimulation, the animal could be made to curve back and forth at will. A similar motion was secured by alternately exposing the dorsal and ventral edges of an animal. As a result, it would swing back and forth, dorsally and ventrally, subject to the frequency of stimulation.

This sensitivity persists in its original strength after the intersiphonal ganglion has been removed, showing its independence of central control. That we are not dealing here with a case of direct stimulation of the muscular tissue (Parker, 1916) is shown by its behavior under chloretone anesthesia. When a *Ciona* is placed in a solution of chloretone in sea water it loses this local photic sensitivity simultaneously with its irritability to touch and to another type of light stimulus which will presently be shown to be undoubtedly mediated by a nervous mechanism. In all probability there is present here a neuromuscular complex similar to the nerve net of lower invertebrates, which is sensitive to light. Evidence for the existence of such a nerve net has already been presented by Jordan (1908) on the basis of his work on the mechanical stimulation of *Ciona*.

2. Under ordinary conditions of experimentation, this sensitivity and its local response are entirely masked by the other, more general, reaction to light. When a *Ciona* as a whole is exposed to a source of illumination, it reacts by a vigorous retraction of its siphons (Nagel,

1896). If the intensity of the stimulus is great, this may be accompanied by a shortening of the animal on its long axis. This retraction reflex has already become familiar due to the work of Loeb (1902) and of Jordan (1908) among others (see Hecht, 1918, *b*) especially in its relation to the removal of the ganglion of *Ciona*.

It is the response of *Ciona* by means of the retracting reflex that forms the basis of the experiments which follow.

3. The orange or red pigment spots on the rims of the siphons are a prominent characteristic of ascidians. *Ciona* possesses eight on the oral rim and six on the atrial. These pigmented masses have been called ocelli on the assumption that they are the photoreceptors of *Ciona*.

Experiments designed to ascertain the location of the photic sense organs showed that the anterior pigment spots were not sensitive to light. A beam of light focussed upon them failed to produce the characteristic reaction. Moreover, their complete removal in no way interfered with the sensitivity of the animal. The protocol of Animal 25.1 gives the details of such an experiment.

Animal 25.1.

Time.		
11.00	In dark room.	
2.25	Exposed to light, 120 candle power, reaction time	3.5 sec.
2.35	" " " 120 " " " "	3.3 "
2.44	" " " 120 " " " "	3.3 "
2.50	Dirty translucent test of animal removed.	
4.30	Exposed to light, reaction time	1.7 sec.
4.37	" " " " "	1.4 "
4.44	" " " " "	2.2 "
4.50	Rims cut away to remove the ocelli of both siphons.	
7.41	Exposed to light, reaction time	1.4 sec.
7.49	" " " " "	1.6 "
8.00	" " " " "	2.0 "
10.00	" " " " "	1.9 "

Of fourteen animals operated in this way, two were killed accidentally. The remaining twelve all gave the same results as the one presented in detail. It is clear, therefore, that these pigment spots are not photoreceptive organs (cf. Hecht, 1918, *b*).

It has already been shown that although the general body surface is sensitive to light, the resulting activity is a purely local one. Gen-

eral body sensitivity could not therefore account for the retraction reflex. By exploring the body with a small beam of light, I found that the seat of this photic sensitivity was in the small area occupied by the intersiphonal ganglion and its related structures. When light was focussed on this spot alone the reaction given by *Ciona* was in all particulars the same as when the animal as a whole was illuminated. No other region of the body possessed this property. As an example of many similar experiments the record of Animal 30.5 is given.

Animal 30.5.

Time.	Part stimulated.	Reaction time.
		sec.
11.40	Entire body.	13.1
11.45	Oral ocelli.	No reaction.
11.50	Ganglion region.	5.0
11.58	" "	5.2
1.57	" "	5.8
2.08	Atrial ocelli.	No reaction.
8.15	Ganglion region.	4.8

It will be noticed that the reaction time to stimulation of the ganglionic region is less than for the exposure of the entire body. This is because the light used for spotting the ganglion region was concentrated to a small area by means of a lens. It will be shown presently that the reaction time varies inversely as the intensity of the light.

It is difficult to say which of the several organs composing the neural mass contains the photoreceptors. Experiments with the opaque *Ascidia atra* of Bermuda (Hecht, 1918, *b*) have shown that its photic sensitivity may be demonstrated only by stimulating the dorsal wall of the *inside* of the oral siphon below the ring of tentacles. This corresponds to the position of the dorsal tubercle, a structure which, in *Ciona*, is closely affiliated with the ganglion. However, though I performed many operations involving the removal of the entire neural mass, as well as only portions of it, the results were of rather doubtful interpretation.

The difficulty probably lies in the fact that a disturbance in the integrity of the neural complex interferes with the conduction of im-

pulses in this region (Fröhlich, 1903). Consequently it was impossible to make a finer localization of the sense organs responsible for the general photic reaction. The presence of the sense organs in the definitely circumscribed region of the neural mass, however, is decisive and clear cut. In a medium sized individual this structure occupies a volume of about 1 cc. or less. In addition it should be pointed out that this area of sensitivity is colorless and contains no apparent pigment of any kind (cf. Wenrich, 1916, p. 306). For the most part the structures are transparent; the ganglionic mass itself, however, is glistening white.

III. Analysis of Reaction Time.

1. The appearance of the retraction reflex as a result of photic stimulation possesses one very important property. This is the presence of a reaction time representing the interval from the beginning of the exposure to light up to the moment when the retraction takes place. This interval may be measured accurately with a stop-watch, first, because it is of rather long duration, and second, because the appearance of the retraction reflex is sudden and clear cut.

The reaction time of any individual *Ciona* to a given intensity of light shows almost no variations from a constant quantity. In Table I are given the reaction times of three animals to the same intensity of light taken at different times of the day. When not exposed to the light, the animals were all kept in a dark room. From these figures it will be seen that the reaction time represents a quantity which may be reproduced under a given set of conditions.

TABLE I.

Light Intensity, 4,880 Candle Meters.

Animal 3.1		Animal 3.2		Animal 3.3	
Time.	Reaction time.	Time.	Reaction time.	Time.	Reaction time.
	sec.		sec.		sec.
1.56	2.8	1.57	1.9	1.59	3.6
4.27	3.2	4.28	2.1	4.30	3.9
9.12	2.9	9.14	2.0	9.15	3.5

2. Before this study had proceeded very far it became clear that the reaction time could not be reduced below approximately 1.5 seconds. No matter how intense the stimulating light, this interval was always maintained. To explain this, I adopted the provisional hypothesis that the reaction time was not a simple interval but that it contained at least two components. This indeed, was soon found to be true, when experiments were made to determine how much of the reaction time was actually devoted to the reception of the photic stimulus and how much was concerned with a real latent period.

By exposing an animal for a shorter time than its reaction time, an interval was found during which it was not necessary for *Ciona* to be illuminated. At the end of this interval it reacted as usual although at the time of reaction, like the hydroids used by Loeb and Wasteneys (1917), they were in the dark. The reaction time is therefore composed of two parts. The first is a sensitization period during which *Ciona* must be exposed to light. The second is a latent period during which *Ciona* need not be illuminated in order to react at the end of the period. With a little practice the sensitization period could be regulated to within 0.2 second. An example is given by Experiment 8.6.

Experiment 8.6. Light Intensity, 743 Candle Meters.

Time.	Time exposed.	Reaction time.
	sec.	*sec.*
8.34	3.6	3.6
9.18	3.5	3.5
10.00	2.0	3.5
10.35	1.0	No reaction.
10.58	1.5	" "
11.32	1.5	" "
12.02	2.0	3.7
1.19	2.0	3.4

Result: Sensitization period = 2.0. Latent period = 1.5.

With the duration of the sensitization period we shall be concerned later, because it varied with the intensity of the light. The latent period, however, showed itself to be a constant quantity under considerable variations in intensity. A series of 53 determinations of

the latent period of nineteen animals gave 1.76 seconds as the average duration of this portion of the reaction time.

3. The latent period must include the time for transmission of the stimulus and kindred phenomena. There is evidence to show, however, that a great portion of it is concerned with a process occurring in the sense organ itself, as is shown in Experiment 9.4.

Experiment 9.4. Light Intensity 4,880 Candle Meters.

Time.	Time exposed.	Reaction time.
	sec.	*sec.*
1.31	2.4	2.4
2.31	2.3	2.3
2.53	0.2	No reaction.
3.24	0.5	7.6
3.41	0.8	3.6
3.47	1.0	2.8
4.22	1.0	2.3
4.43	2.1	2.1

It will be seen that, within limits, the shorter the exposure time (sensitization period) the longer the reaction time, and consequently the latent period. The latent period in the main must therefore take place in a locality where it may be immediately influenced by the amount of light energy received. This would indicate that it is the outward manifestation of the duration of a process within the sense organ itself.

4. The existence of a latent period of nearly 2 seconds is hardly to be considered as an advantage to the life of the species. The ordinary daylight of southern California is deleterious to *Ciona*. The animals die very soon in an aquarium in diffuse daylight. When placed near a window they contract and perish in a day, whereas in a dimly lighted room or in the dark they remain in excellent condition for a week or more. They are normally found on the under side of floats.

The comparatively long delay in responding after a sufficient stimulus has been received cannot thus be of assistance to the animal in its response to a noxious stimulus. It must therefore be attributed to the duration of a chemically necessary process occurring, as suggested, in the sense organ.

IV. Effect of Intensity.

1. In order to study the effect of different intensities of light on the duration of the sensitization period, a single source of light was used. This was a Mazda lamp of 120 candle power. By keeping it at different fixed distances from the animal, six intensities were secured, as given in Table II.

Ten animals varying in size from 3.5 to 10.5 cm. were used for this experiment. It may be said at once that no difference in sensitivity was found for individuals of different size. The reaction time of each animal to each of the six intensities of light was determined three times; thus, eighteen observations were made on each animal.

TABLE II.

1	2	3	4
Intensity (I).	Reaction time.	Sensitization period (t).	$I \times t$
candle meters	*sec.*	*sec.*	
521	11.25	9.49	4,944
743	8.89	7.13	5,298
1,180	5.98	4.22	4,980
2,080	3.59	1.83	3,806
4,880	2.72	0.96	4,685
9,920	2.24	0.48	4,762
Average.....			4,746

The determinations were purposely scattered over a period of 12 hours so that any fatigue effects would be avoided. In Table II, Column 2, are given the average values for the reaction times of the ten animals. Each figure represents an average of 30 determinations. In addition the data are plotted in Curve A of Fig. 1. From both these sources it is apparent that the reaction time varies inversely as the intensity. In Column 3 are given the values for the sensitization periods corresponding to the different reaction times. These were calculated by subtracting the duration of the latent period, 1.76 seconds, from the reaction time values.

2. On the supposition that the sensitivity of *Ciona* depends upon a photochemical reaction, the relation between the intensity of the

light and the sensitization period should be expressed by the reciprocity law as found by Bunsen and Roscoe (1862). This states that for a given photochemical effect (E) a definite quantity of energy is required, and this same result is obtained whether the intensity (I) is high and the exposure (t) short or conversely. In other words,

$$E = k \cdot I \cdot t \quad (1)$$

The applicability of this law has been demonstrated by Blaauw (1909) and by Fröschel (1909) for the photic reactions of some plants, and by Loeb and his coworkers for the orientation of several species of animals. In *Ciona*, its existence would be demonstrated if the product of the intensity and the sensitization period were a constant quantity. In Column 4 of Table II are given the values obtained. It will be seen that the figures agree well with one another and with the average value.

The matter may be illustrated graphically. The equation representing the Bunsen-Roscoe law may be written

$$\frac{E}{t} = k \cdot I \quad (2)$$

In this form, it represents the equation of a straight line. Since, in *Ciona*, E is constant, $\frac{E}{t}$ may be represented by the reciprocal of the sensitization period, and the relation between this quantity and the intensity should be represented by a straight line. Such indeed is the case, as shown by the position of the points in Curve B of Fig. 1.

3. In terms of the processes that go on in the sense organs, the applicability of the reciprocity law means that during the sensitization period the light must form a constant quantity of a substance before it can produce its stimulating effect. Undoubtedly this involves the conversion of a photosensitive substance into something else which acts as an "inner stimulus." Many such photosensitive substances are well known and have been studied with considerable care (Sheppard, 1914).

With a more precise presentation of the dynamics of the photochemical reaction in *Ciona* we shall deal in a following section. At present, however, I wish to emphasize a corollary to the fact that the

photic reaction proceeds according to the Bunsen-Roscoe rule as indicated in equation (1). It is, that, with a given intensity of illumination, the sensitization period indicates the quantity of stimulus received by the animal. The photochemical effect (E) will vary with the duration of the exposure (t) when the intensity (I) is constant.

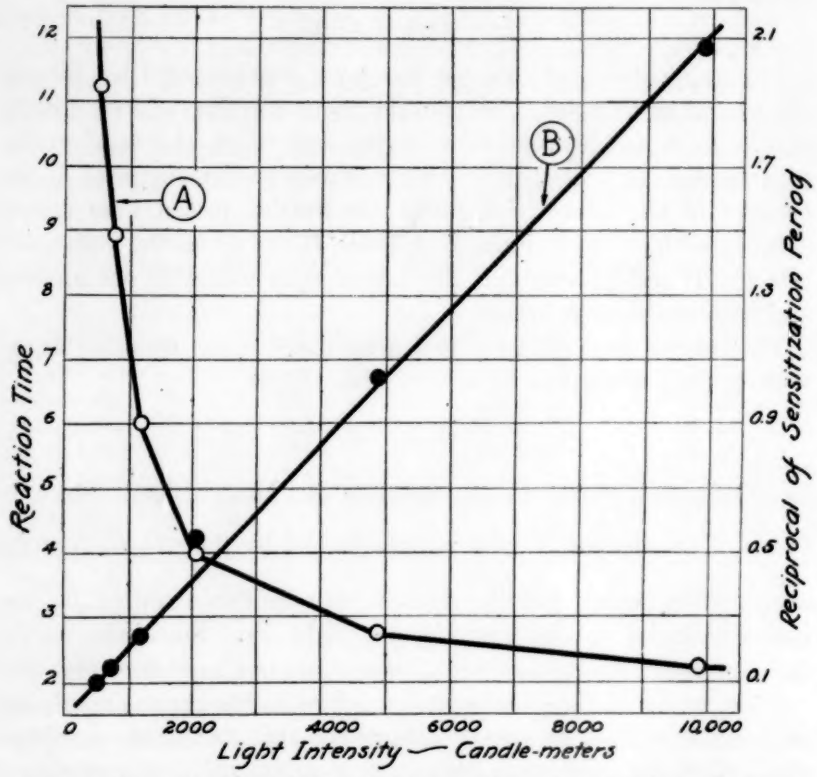

FIG. 1. Curve A gives relationship between intensity of light and reaction time of *Ciona*. This curve is an hyperbola as is shown by Curve B representing the relation between the reciprocal of the sensitization period and intensity.

The reaction time at any moment may therefore be used as a measure of the amount of photochemical effect necessary in order to act as a stimulus in the sense organ. In this way we may investigate the sensitivity of *Ciona* under different conditions in order to gain a clearer insight into the photochemical process which underlies it.

V. Dark Adaptation.

1. Under ordinary circumstances, *Ciona* remains expanded in diffuse light. The stream of sea water flows steadily through its branchial sac except for certain rhythmic interruptions presented in detail elsewhere (Hecht, 1918, *a*). Similarly, the animal becomes non-responsive after a time when placed in a lower or a higher intensity of illumination. After such adaptation it fails to react to a decrease in the intensity of the illumination but responds vigorously to an increase. On remaining for some time in the dark—becoming "dark adapted"—it is sensitive to the light of an ordinary Mazda lamp.

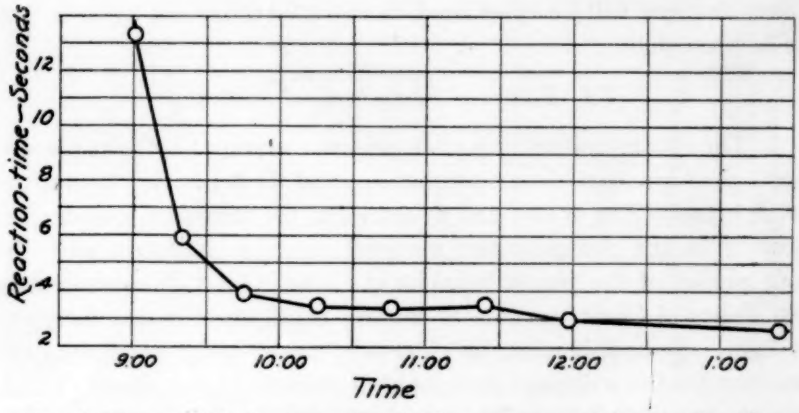

FIG. 2. Course of dark adaptation of *Ciona* represented by reaction time at different periods of sojourn in dark room.

The process of dark adaptation was followed in some detail. Animals which had been in the light for a few hours were removed to the dark room and their responses to light noted at regular intervals. A constant intensity was used, so that any variations in the reaction time would serve to indicate corresponding variations in the quantity of stimulating agent required to cause a reaction.

The results were regular and fairly uniform. The process of dark adaptation required at least 2 hours; usually more. At first the reaction time was long, then it shortened rapidly, then more slowly, and finally at the end of 2 or more hours it reached a constant minimum. A good example of what occurred is given by Fig. 2. The points on

the curve represent the averages of the reaction time of two individuals which were used in this particular experiment.

At any given point, the duration of the reaction time (really of the sensitization period) measures the quantity of light energy required by the photochemical system to produce a response of *Ciona*. In terms of the Bunsen-Roscoe law this, in addition, measures the photochemical effect, and consequently the amount of photosensitive substance which must be changed before the retraction reflex can be elicited. The curve in Fig. 2 shows this quantity to be at first large, then smaller and smaller. At the end of the process of dark adaptation it becomes constant at its minimal value. The interpretation of these changes will be considered in the photochemical system now to be presented.

VI. Nature of Photochemical System.

1. The decomposition of a photosensitive material by light presupposes the formation of such a substance and its deposition within the sense organ. The nature and dynamics of the formation and decomposition of the photosensitive material must be consistent with the four phases of the photic behavior of *Ciona*. First, it must explain the ability of *Ciona* to come to sensory equilibrium in any intensity of light. Second, it must be clear why under given conditions the reaction time to a definite increase of illumination is constant. Third, the process must be consistent with the Bunsen-Roscoe rule for the relation between intensity and sensitization period. Fourth, it must indicate the basis of the regular change in the reaction time that occurs during dark adaptation.

2. The simplest assumption which will account for all the phenomena observed is that we are dealing here with a reversible chemical reaction. A photosensitive material (S) is formed from its precursor (P), the reaction being of the first order and following the ordinary laws of such processes. It may be of a higher order, but the assumption is made for the sake of simplicity. The action of light upon the photosensitive material (S) is to change it back into its precursor (P). In accordance with current photochemical knowledge such a reaction will follow the Bunsen-Roscoe rule. A reaction which in many respects resembles the system suggested here, is the conversion of

anthracene into dianthracene by light, and its reversal in the dark (Sheppard, 1914, p. 214).

Let us consider the reaction which occurs normally, and of course in the dark, and which is supposed to regenerate the photosensitive material from its precursor ($P \rightarrow S$). The curve in Fig. 3 indicates the process on the basis of the ordinary velocity curves for chemical reactions. The formation of the photosensitive material (ordinates at the right) proceeds at first rapidly, then it loses speed gradually, and finally it reaches a point of equilibrium which represents a definite ratio between the concentration of the sensitive material and its precursor. The important thing to be noticed in the curve is the change

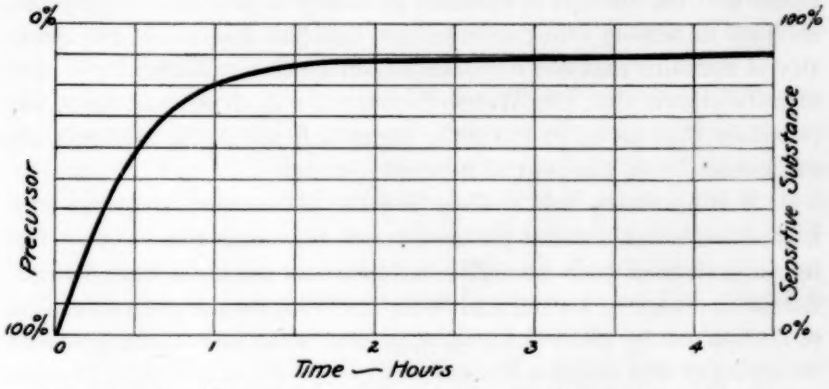

FIG. 3. Hypothetical curve of reaction $P \rightarrow S$ based on data of Fig. 2.

in the quantity of precursor (ordinates to the left) during the reaction. At first the amount of this substance is large, then it decreases rapidly, then more slowly, and finally it reaches a constant minimum. On comparing this rate of change with the one undergone by the reaction time during dark adaptation it is seen that the two run quite parallel. It has been assumed that the action of the light during the sensitization period causes the photosensitive material to be changed into its precursor. This means that the variation in the amount of the precursor formed at the successive reactions during dark adaptation is in general similar to the change in the amount of precursor still unused in the chemical reaction. From the relationship between these two variables we must conclude that, in order to act as a stimu-

lus in the sense organ, the quantity of precursor formed by the stimulating light bears a definite ratio to the amount already present. As a matter of fact the curve in Fig. 3 is not a theoretical one, but was actually constructed from the data of Fig. 2, on the assumption that the ratio between the precursor formed and the precursor already present is as 1: 10. The fact that it resembles the ordinary velocity curves for chemical reactions strengthens the force of the above explanation.

The necessity for the acquisition of a constant ratio between precursor formed and precursor present, before stimulation can occur, is essentially the requirement of the Weber-Fechner concept. This states that the amount of stimulus necessary to produce a perceptible increase in sensory effect represents a constant fraction of the quantity of stimulus that has previously been applied. Waller (1895) has already shown that the Weber-Fechner law is dependent upon the processes that go on in the sense organ and not in the transmitting mechanism or in the central nervous system.

3. It is necessary now to show how the photochemical system suggested above can account for the sensory phenomena in *Ciona*. The investigations of such reversible reactions as proposed here, in particular the work of Luther and Weigert (1905) on the polymerization of anthracene by ultra-violet light, give us some idea of the processes occurring in our reaction system:

$$P \underset{\text{light}}{\overset{\text{"dark"}}{\rightleftharpoons}} S$$

The stable condition is that of the sensitive material S in the dark. Starting with the precursor P in the dark, it changes spontaneously to S. Light converts S back into P. However, because the reverse change, $P \rightarrow S$, is independent of light, it will be resumed as soon as some P has been formed by the light. Moreover, the more precursor (P) that is formed by the light, the greater will be the rate at which the photosensitive substance will be formed. This follows from the ordinary mass action principle. At a given intensity of illumination the two reactions will proceed in opposite directions at an equal rate, and a "stationary condition" simulating a chemical

equilibrium will be reached, in which the quantity of precursor will be constant. This will happen at any intensity of light no matter from which direction it is approached.

Constancy in the quantity of precursor means no stimulation of *Ciona*. We have thus a mechanism which possesses the first requirement for an explanation of the photosensitivity of *Ciona*. This, it will be recalled, is the ability of the animal to come into sensory equilibrium at any intensity of illumination. An explanation of the second characteristic of the sensitivity follows from this at once. In order to act as a stimulus the increase in illumination must be maintained for such a time as to produce a definite fraction of the precursor present. This is constant; therefore, the reaction time will be constant. The third requirement is also easily deduced. The quantity of precursor required for stimulation is constant. Since the reaction $P \leftarrow S$ follows the reciprocity law, it will make no difference whether the intensity is low and the sensitization period long or *vice versa*. The variation in the reaction time during dark adaptation has already been dealt with to show that it depends on the suggested photochemical system. The latter therefore completely fulfills our demands for an explanation of the photic responses of *Ciona*.

4. It should be pointed out that the proposed chemical system is the simplest, but not the only hypothesis capable of explaining the sensory phenomena observed in *Ciona*. There are two reactions required: one, a "light" reaction, and the other, a "dark" reaction. It has been assumed that these are the two phases of a reversible reaction system. A somewhat more complex, yet essentially similar hypothesis is to consider the two reactions as forming a catenary system.

According to this alternative suggestion, a photosensitive substance S is decomposed, not into its precursor but into another substance DS. This is the "light" reaction. The decomposition product DS then becomes converted into an inert material M. This is the "dark" reaction. Thus

$$S \xrightarrow[\text{light}]{} DS \quad (1) \qquad \text{and} \quad DS \xrightarrow[\text{"dark"}]{} M \quad (2)$$

Reaction (1) occurs in the light and follows the Bunsen-Roscoe law. The decomposition product (DS) is the stimulating substance, and in

order to act as a stimulus the amount of it to be formed by the light must bear a constant ratio to the amount of *DS* already present in the system. Reaction (2) removes the decomposition product from the reaction system. It possesses the same velocity and properties as the "dark" phase of the reversible reaction system presented before. Its occurrence is represented by the process of dark adaptation shown in Fig. 2, and its course is given by the curve in Fig. 3 in which *P* represents *DS* and *S* represents *M*.

Which of the two suggested photochemical systems comes nearer the truth future work must decide. The present data may be explained in terms of either. The simpler one is therefore provisionally adopted.

VII. Repeated Stimulation.

1. There are several predictions which may be made on the basis of the hypothetical chemical system which has been proposed. My stay at La Jolla, however, limited me to an investigation of but one of these.

We have seen that the "dark" reaction ($P \rightarrow S$) is a comparatively slow one, requiring several hours to reach an equilibrium. Therefore, if a thoroughly dark-adapted *Ciona* is stimulated by light at short intervals, say every minute, practically no new photosensitive material will be regenerated during a period of about 10 minutes. The quantity of precursor however will be continually increased, depending upon the duration of the exposure to light. This follows from the Bunsen-Roscoe rule which has been shown to apply to *Ciona*. The quantity of precursor necessary to stimulate must be a constant fraction of the amount of precursor already present. Therefore, during such a series of regular stimulations, the amount of precursor required must be larger at each succeeding reaction. The rate of increase in the amount of precursor will be logarithmic, each quantity representing the same fraction of the sum of the quantities that have been formed before. The sensitization period measures the quantity of precursor formed at each reaction. Therefore, in such a set of repeated stimulations, the sensitization periods should always bear a definite ratio to the sum of the sensitization periods that have preceded it.

All the experiments made to test this prediction verified it with amazing unanimity. *Cionas* which had been in the dark for several hours were exposed to a light of 2,000 candle meters at regular intervals of 1 minute and the reaction time at each exposure noted. The results of four experiments are given in Figs. 4 and 5.

The upper curve in Fig. 4 represents the actual data, the moments of stimulation being the ordinates and the sensitization periods the abscissæ (upper scale). The curve has the usual Weber-Fechner appearance, its logarithmic nature being shown in the lower curve of the

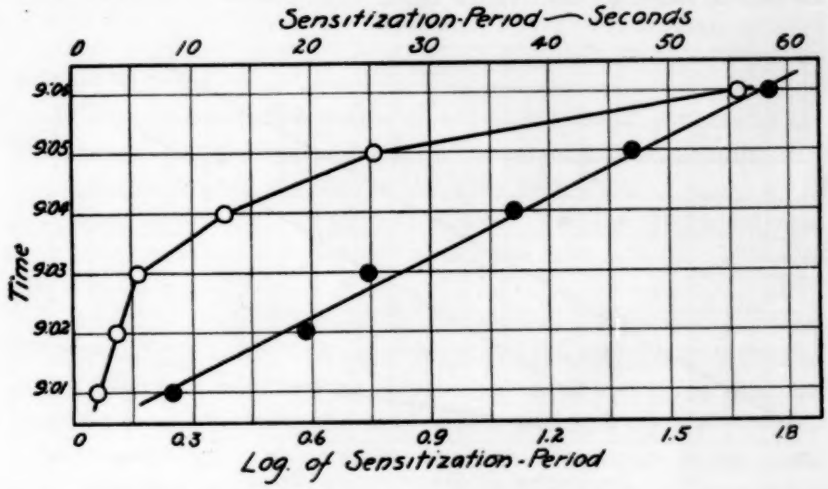

FIG. 4. Upper curve gives sensitization periods of a *Ciona* during regularly repeated stimulation at minute intervals. Its logarithmic nature is shown by the lower curve which gives the logarithms of the same sensitization periods.

same figure. The lower curve has the same ordinates, but the abscissæ are the logarithms of the sensitization periods (lower scale). That the points form a straight line is undoubted.

In Fig. 5 are given the data for three other animals. The logarithms of the sensitization periods are plotted as before, and in each case the points very clearly form a straight line.

This series of experiments therefore is entirely consistent with the three cardinal features of the hypothetical chemical system proposed as an explanation for the photic sensitivity of *Ciona*. These are, first,

that the "dark" reaction of sensitive material regeneration is comparatively slow; second, that the "light" reaction changes the sensitive material into its precursor according to the Bunsen-Roscoe law; and third, that in order to act as a stimulus, the light must form such a quantity of precursor that it will bear a definite ratio to the amount of precursor already present.

2. There is a phase of these data on regularly repeated stimulation to which attention has already been called (Hecht, 1918, *c*) and which must be emphasized. The individual experiments were ended when the animal failed to react to the light. The course of an experiment

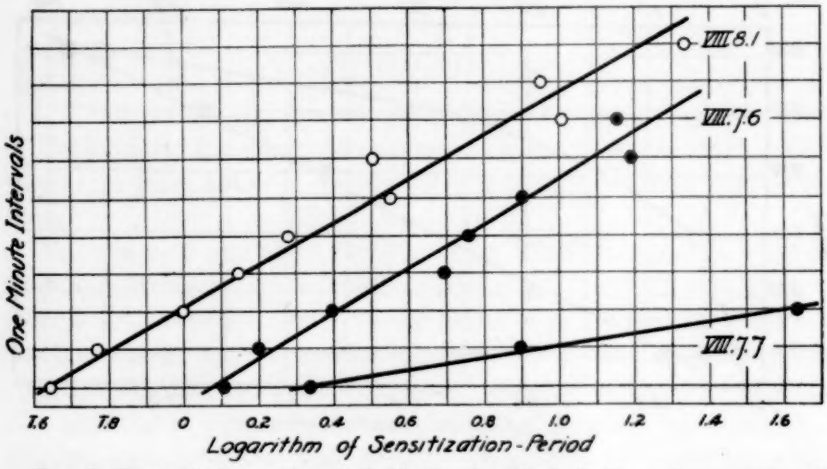

FIG. 5. Data of three other individuals plotted similarly to lower curve of Fig. 4.

was therefore as follows. The reaction time to the same intensity became larger and larger and finally the animal ceased to react,—because "adapted" to the stimulus. This is the kind of evidence which has been used to prove the existence of a "higher behavior" in lower animals (Kinoshita, 1910). According to this line of reasoning, the increased reaction time represents a process of learning, and the complete cessation at the end a condition of "indifference" to the stimulus, or a state of adaptation.

I hope that the experiments presented in this communication will show the futility of such merely verbal explanation. From the data

given here it seems clear that the entire process takes place in the sense organs themselves. The attainment of a condition of sensory equilibrium of *Ciona* depends upon the existence of a "stationary condition" of the reversible reaction system responsible for its photosensitivity. Dark adaptation represents such a sensory equilibrium when the "light reaction" is completely removed. Rapid adaptation to light is essentially the reverse process, in which the "dark" reaction is practically absent, and the "light" reaction is in full swing.

In these experiments there is no need either for the postulation of a learning process or for the presence of a "higher behavior" in order to rationalize the results obtained.

SUMMARY.

1. *Ciona* possesses two means of responding to an increase in the intensity of illumination. One is by means of a local reaction; the other is by a retraction reflex of the body as a whole.

2. The "ocelli" are not photoreceptors. The photosensitive area is in the intersiphonal region containing the neural mass. This area contains no pigment.

3. The reaction time to light is composed of a sensitization period during which *Ciona* must be exposed to the light, and of a latent period during which it need not be illuminated in order to react to the stimulus received during the sensitization period.

4. The duration of the reaction time varies inversely as the intensity. Analysis shows the latent period to be constant. The relation between the sensitization period and the intensity follows the Bunsen-Roscoe rule.

5. During dark adaptation the reaction time is at first large, then it decreases until a constant minimum is reached.

6. A photochemical system consisting of a reversible reaction is suggested in order to account for the phenomena observed. This system includes a photosensitive substance and its precursor, the dynamics of the reaction following closely the peculiarities of the photosensitivity of *Ciona*.

7. It is shown that in order to produce a reaction, a constant ratio must be reached between the amount of sensitive substance broken down by the stimulus and the amount previously broken down.

8. From the chemical system suggested certain experimental pre-

dictions were made. The actual experiments verified these predictions exactly.

9. The results obtained with regularly repeated stimulation not only fail to show any basis for a learning process or for the presence of a "higher behavior," but follow the requirements of the photochemical system suggested before.

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AN INDICATOR METHOD OF MEASURING THE CONSUMPTION OF OXYGEN.

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(Received for publication, September 11, 1918.)

The study of respiration has been greatly facilitated by using indicators to measure the production of carbon dioxide. It is desirable to employ similar methods for estimating the amount of oxygen consumed in respiration. With this in view the writer has investigated a number of substances, some of which appear to give promising results. The purpose of the present note is to describe very briefly the use of *Limulus* blood for this purpose.

The blood of *Limulus* (and of a number of other invertebrates) when shaken up with air absorbs oxygen and turns blue, but on standing is reduced and in consequence becomes decolorized. It has been pointed out by Alsberg[1] and by Harvey[2] that the decolorization is due (in large part at least) to the activity of microorganisms. It seemed to the writer that this might be utilized to measure the rate of consumption of oxygen in respiration.

The procedure adopted is as follows: Large horseshoe crabs are taken and an incision[3] is made at the joint in the middle of the body; the body is then repeatedly bent back and forth to expel the blood. After the blood has stood for a short time a clot forms from which the clear blue serum may be poured off. To a portion of this sufficient ether is added to form a saturated solution; this is allowed to stand in a stoppered bottle with occasional gentle shaking, until the ether is dissolved. A number of glass tubes are prepared by sealing

[1] Alsberg, C. L., *J. Biol. Chem.*, 1915, xxiii, 495. Alsberg, C. L., and Clark, E. D., *ibid.*, 1910–11, viii, 1. Alsberg, C. L., and Clark, W. M., *ibid.*, 1914, xix, 503. Alsberg, *ibid.*, 77.

[2] Harvey, E. N., *J. Gen. Physiol.*, 1918, i, 133.

[3] This enters the heart cavity. It is desirable to cleanse and dry the surface around the joint before making the incision.

one end and fitting to the other a piece of rubber tubing which can be clamped shut. Various dilutions of the blood are made (with a saturated solution of ether in sea water), each of which is placed in a separate tube. In one tube undiluted blood is placed. All the tubes are clamped shut to prevent the evaporation of ether.

The ether prevents the decolorization of the blood and the various dilutions furnish shades of color which serve as standards in subsequent experimentation.

If fresh water organisms are used in the experiments the procedure must be modified. Since the salt content of the blood is approximately that of sea water it must be reduced by dilution or by dialysis. If this is carried beyond a certain point precipitation occurs and the color of the aerated blood is paler in consequence.

The organisms are placed in glass tubes, provided with rubber tubing as described above, and blood (diluted or undiluted) is added; the tubes are then clamped shut, taking care to exclude bubbles of air. The observer then notes the time required to produce a definite change[4] in color (as determined by comparison with the tubes containing blood to which ether has been added).[5] It is desirable to observe the tubes against a dark background, preferably while facing the source of light. The best results are usually obtained by viewing the tubes from the end. For this purpose the tubes are inverted so that the rubber tubing is below; the organisms sink into it and the rubber tubing may be temporarily pinched off above the organisms so they cannot be seen.

As soon as a definite change in color is observed the tubes containing the organisms may be opened and shaken with air so as to restore the original color. There must be a control tube containing blood without organisms or ether; if this control becomes decolorized the experiment must be rejected, unless the decolorization is so slow as not to interfere with the result. Freshly drawn blood does not decolorize except very slowly; this is also true of freshly dialyzed blood, and of blood heated[6] for 5 minutes to 60°C. Blood preserved

[4] The tubes should be shaken from time to time.

[5] A "Daylight" lamp was used in most of the experiments.

[6] Heating at lower temperatures (40–55°C.) for a longer time may be preferable.

by the addition of ether may be freed from ether by a current of air[7] and does not decolorize rapidly until it has stood for some time. Blood which has been carefully dried to a jelly-like mass may be kept in this condition; it dissolves readily on the addition of water. But if dried to a hard, brittle mass it does not readily dissolve.

Under favorable conditions blood containing certain organisms,[8] (such as bacteria, or young *Limulus* in the trilobite stage) is quickly decolorized (in some cases within 2 minutes) and the time required for decolorization is remarkably constant.[9]

When the normal rate of oxygen consumption has been established by repeated determinations, reagents may be added and changes in the rate may be determined. In this way the relative rate under the influence of the reagent may be ascertained without knowing the absolute amounts of oxygen consumed in either case.

A variety of other substances, such as indigo-carmine, methylene blue, malachite green, etc., are also reduced and it is possible that some of them may prove more useful than *Limulus* blood. Experiments on this subject are being continued.

SUMMARY.

The blood of the horseshoe crab (*Limulus*) absorbs oxygen and turns blue when shaken in air. In the presence of certain organisms which consume oxygen it is quickly decolorized. By measuring the time required for the change of color the rate of consumption of oxygen may be determined.

[7] The air should be filtered or washed to remove bacteria before entering the blood.

[8] The salt content must be adjusted to the needs of the organism; it may be quickly ascertained by titration.

[9] The normal rate is sometimes irregular, due to factors not fully understood. In these cases the results are rejected. The disturbing factors require further study.

COMPARATIVE STUDIES ON RESPIRATION.

I. Introduction.

By W. J. V. OSTERHOUT.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication September 6, 1918.)

In the course of studies on antagonism the writer made experiments on the action of antagonistic substances on respiration. As a result of his experiments he became dissatisfied with the existing means of studying respiration in plants and undertook to devise more satisfactory methods. At his suggestion Dr. Haas developed an indicator method[1] which proved to be very satisfactory for aquatic organisms. However, it had serious limitations which were subsequently removed by a method devised by the writer. The new method is accurate, rapid, and convenient, and can be used for organisms of all kinds.[2]

A series of studies has been made in the writer's laboratory by the use of these methods, the first of which are here brought together. The work of Mr. Gustafson deals with higher fungi, that of Mrs. Brooks with bacteria, that of Miss Thomas with flowering plants, and that of Miss Irwin with a variety of animal material. The work of Haas[3] on the marine alga *Laminaria* was carried out under the same conditions, and may therefore be compared with the results described in this series.

In the first investigations the technique described by Haas[1] was employed in most cases. Phenolsulfonephthalein was added to the liquid containing the organisms, and respiration was allowed to proceed until a definite change in acidity had occurred. Usually the organism was then placed in a fresh sample of the solution and the process was repeated until the normal rate of respiration was ascer-

[1] Haas, A. R., *Science*, 1916, xliv, 105.

[2] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918, i, 17.

[3] Haas, *Science*, 1917, xlvi, 462; *Proc. Nat. Acad. Sc.*, 1917, iii, 688.

tained;[4] the reagent was then added and new measurements of respiration were made in the same manner as before.

The rate of respiration after the addition of the reagent is expressed in each case as per cent of the normal rate. The normal rate is usually determined by taking the average of successive determinations. The normal rate may be different in each experiment (on account of differences in the quantity or kind of material used), but it is in all cases taken as 100 per cent.

The relative rate was ascertained in all cases by comparing the times required to produce a definite change of acidity and not by a comparison of the changes in acidity produced in equal times. The former method compares the reaction velocities directly, while the latter may not.[5]

This method of comparison has the further advantage of making it unnecessary to know what change in pH value is produced by adding a definite amount of CO_2. For if we always start the measurement from the same pH value and carry it to the same end-point, we can be certain that the same amount of CO_2 has been produced in each case, although we may not know what this amount is. It is, in fact, quite unnecessary to know it if we are comparing the times required to do equal amounts of work.

An additional advantage of this method is that an error in the buffer solutions does not affect the results, providing we use the same buffer solutions throughout. For this purpose they are made up in large quantities.[6] Two solutions are chosen, the pH value of one being taken as the starting point (to which the experimental solution is always brought when starting a measurement) and that of the other as the end-point (to which the experimental solution comes as the result of respiration).

Certain precautions which are essential to accurate work may be briefly mentioned, in addition to those described by Haas.[3] It is necessary to use the purest reagents and in particular to see that they are neutral (or practically so); stock solutions may be kept in Pyrex flasks or tubes to avoid alkalinity due to the glass. Slight departure

[4] Unless the normal rate was fairly constant the experiment was rejected.

[5] Osterhout, *Science*, 1918, xlviii, 172.

[6] Buffer solutions of phosphates or borates will keep for a long time.

from neutrality may be compensated by adding CO_2 to the tap water or removing CO_2 (by means of a current of air free from CO_2 or by a current of hydrogen). In this way the solution may be brought to the most convenient pH value; all experiments are then started at this value. It is desirable to choose a value in the region where the indicator is sensitive.

In case alkali is added to bring the solution to a given pH value it must be done before the normal respiration is measured, so that the buffer effect of the alkali will be the same in the measurements of normal and abnormal respiration.

The buffer effect of all reagents must be carefully measured. This may be done by means of an apparatus recently described.[7] In the case of the reagents employed in these studies there is practically no buffer effect (unless the contrary is expressly stated). The result of buffer action is to make the amount of respiration appear smaller than it really is.

It was found that no alkali or acid (other than carbonic) was given off by the organisms studied. This was determined by placing the organisms in tap water free from CO_2 and allowing them to respire for some time so as to change the color of the added indicator; the CO_2 was then driven off (by a current of air free from CO_2) and it was found that the indicator returned to its original color.

It was found by control experiments (in which the indicator was added after respiration took place) that the indicator itself (at the concentrations employed) had no effect upon respiration.

The accuracy of the measurements depends on developing skill in matching standard colors under uniform conditions. The standard colors are prepared by making a series of buffer mixtures[8] containing the same concentration of indicator as the experimental solutions and contained in Pyrex tubes of the same size as those in which the organisms are respiring. Various devices may be employed to make the matching more accurate. A background of white or light gray paper is used by many, while others prefer opal or ground glass. In some cases it is advisable to screen off the lower part of the tube or

[7] Osterhout, *J. Biol. Chem.*, 1918, xxxv, 237.

[8] Cf. Osterhout, W. J. V., and Haas, A. R. C., *Science*, 1918, xlvii, 420, footnote 7. Frequent renewal of the buffer solutions is advisable.

to place the tube in a hole bored in a block of wood and to view it through another hole bored at right angles to the first. The last method is very useful when there is a color (due to small suspended organisms or to the giving off of coloring matter by the organism) which is superimposed on that of the indicator. This color is contained in the tube which is put into the wooden block in line with the tube containing the buffer solution, so that the light passes through both tubes before reaching the eye. In this way the color of the indicator can be varied independently of the disturbing color, which is due to the organism.

Uniform conditions for comparison of colors were secured by the use of the "Daylight" lamp.[9] This gives a uniform source of light under which colors can be matched with sufficient accuracy, so that the investigator is free from disturbances due to fluctuations of daylight.

The best test of the accuracy of the work is to make repeated measurements on the same material in its normal environment at constant temperature. Tests made with favorable material show that the variation is very small, the probable error (in the time required to produce a standard change in acidity) being less than 1 per cent. Hence if greater variations are found, they must be due to the variability of the material, to the personal equation of the observer, or to unfavorable conditions (*e.g.* of illumination) in comparing colors, rather than to the method. A probable error of less than 1 per cent must be regarded as highly satisfactory for biological measurements.

The results obtained by the use of the new apparatus[2] are quite as accurate as those obtained by the original method.[1]

Attention may be called to two things in respect to the curves: (1) After each measurement there is an interval during which the solution is changed; since the organism is exposed to the action of the reagent during this interval the time should be included in the total time of exposure (as shown in Fig. 1). (2) The rate is obtained by taking the reciprocal of the time required to produce the standard

[9] Cf. Luckiesh, *Science*, 1915, xlii, 764. The form used is known as "north light."

change in acidity; hence it represents the average rate for that period. If the rate changes during the period, the average rate is probably the actual one somewhere near the middle of the period. Hence in Fig. 1, each ordinate representing the average rate is placed in the middle of the period. In these papers all curves in which rate is

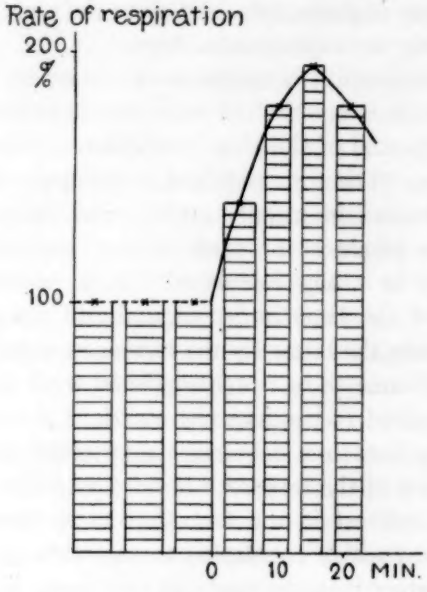

FIG. 1. Curve showing the rate of respiration under normal conditions (dotted line), and under the influence of a reagent (unbroken line). The periods during which measurements are made are indicated by horizontal shading; the intervals during which no measurements are made are left blank. Each measurement gives an average rate for the period; as this average rate is probably the actual rate near the middle of the period, the ordinate expressing the average rate is placed in the middle of the period. For convenience in comparison, time is reckoned from the beginning of exposure to the reagent.

plotted against time are made in this manner. For purposes of comparison the time expressed on the abscissæ is usually reckoned from the beginning of exposure to the anesthetic. The preceding part of the experiment, during which respiration was going on under normal conditions, is also represented on the curve but in this part the

figures expressing time are omitted from the abscissæ in order to avoid confusion.

In averaging curves the following procedure was adopted. A curve was drawn for each experiment (plotting rate of respiration as ordinates and time as abscissæ). These curves were averaged graphically, making use of interpolated points. It may be stated that this procedure is perfectly sound since the error of interpolation is usually less than the experimental error.

The rate of respiration is stated in all cases as per cent of the normal rate (which is always taken as 100 per cent). The rate is taken as the reciprocal of the time required to produce the standard change in acidity. The time required to produce this change under normal circumstances is usually stated, and from this the actual time required to produce the same change under the influence of the reagent may be easily calculated. It is not necessary to find the reciprocals of the time under normal and abnormal conditions and then to divide the latter by the former in order to get the relative rate; for the same thing is accomplished more quickly by dividing the time required to produce the standard change under normal circumstances by the time required to produce the same change under the influence of the reagent.

Of particular interest is the fact that these investigations were made by a method which enables us to ascertain the time curves of respiration by determinations made at very brief intervals (in some cases 1 minute or less). When the intervals are long (as in practically all previous investigations) it may happen that a rise in the rate of respiration which is quickly followed by a fall cannot be detected; and in general the form of the curve cannot be determined with sufficient accuracy to enable us to study the dynamics of the process.

Another point of importance is that so wide a range of material was studied that it is possible to judge whether the results obtained are of general validity. The plants included bacteria, the higher fungi, algæ, and flowering plants, while the animals included insects, frog eggs and tadpoles, and fish embryos. Precisely similar experiments were made on all these forms and as these experiments were all carried out under the same conditions the results are comparable in all respects.

All of these papers deal primarily with the effect of anesthetics on respiration. The special interest of this problem is too well known to require extensive discussion. Of late it has centered largely about the theory of Verworn, which states that anesthesia is a kind of asphyxia, due to a checking of respiration by the anesthetic.

Although this theory has been widely accepted there are excellent reasons for regarding it as invalid. Among these may be mentioned the observations of Warburg,[10] who found that phenylurethane inhibited cell division in the sea urchin egg without altering the consumption of oxygen, and those of Winterstein[11] who found that anaerobic animals are easily narcotized, which is difficult to understand if narcosis depends on interference with oxygen consumption. Loeb and Wasteneys[12] showed that to produce complete narcosis in *Fundulus* embryos by lowering the rate of respiration (by means of KCN) it was necessary to diminish respiration to one-fourteenth of the normal; but the same degree of narcosis could be produced by chloroform with a lowering of respiration amounting to only 5 per cent (or even less). In this case it would appear that anesthesia is not due to the checking of respiration by the anesthetic but to some other mode of action. This conclusion was confirmed by a variety of experiments made with other anesthetics and upon other organisms. Winterstein[13] in a later paper has shown that in the spinal cord of the frog, anesthetized with alcohol, the rate of respiration is above the normal.

The experiments upon plants made by various observers[14] are not in agreement. This is doubtless due to differences in the method of experimentation. One point of great importance which has been brought out in recent studies, particularly by those in this series,

[10] Warburg, O., *Z. physiol. Chem.*, 1910, lxvi, 305.

[11] Winterstein, H., *Biochem. Z.*, 1913, li, 143.

[12] Loeb, J., and Wasteneys, H., *J. Biol. Chem.*, 1913, xiv, 517; *Biochem. Z.*, 1913, lvi, 295.

[13] Winterstein, *Biochem. Z.*, 1914, lxi, 81.

[14] For a review of the literature see Czapek, F., Biochemie der Pflanzen, Jena, 2nd edition, 1913, i, 195 ff. See also Ewart, A. J., *Ann. Bot.*, 1898, xii, 415, and Appleman, C. O., *Am. J. Bot.*, 1916, iii, 223. For a general review of the literature on animals, see Winterstein, H., *Biochem. Z.*, 1913, li, 143; also Höber, H., Physikalische Chemie der Zelle und der Gewebe, Jena, 4th edition, 1914, 460 ff.

is that the result depends in most cases on the length of the experiment. This is due to the fact that under the influence of the anesthetic the rate of respiration constantly changes. In the older experiments this was overlooked and the discrepancies in the results are doubtless due in large measure to this circumstance. It is important to be able to determine the rate of respiration at intervals of 5 or 10 minutes, or even less, as is possible by the method employed in these studies.

All of the plants studied in the writer's laboratory (including representatives of bacteria, higher fungi, algæ, and flowering plants) agree in their behavior toward anesthetics. While lower concentrations produce no effect on respiration, higher concentrations cause a rise followed by a fall. In general the rise of respiration appears to be associated with reversible anesthesia while the fall below the normal rate indicates irreversible toxic effects, at least if it goes much below the normal.

It is evident that these results are directly opposed to the theory of Verworn.

The results obtained by Miss Irwin upon animals are likewise not in agreement with Verworn's doctrines, for although they show that anesthetics may produce a temporary decrease of respiration, which cannot be wholly explained on the ground of cessation of movement, this decrease is much too small to produce anesthesia.

On the other hand Miss Irwin's observations are interesting as showing an apparent difference between animals and plants in their behavior toward anesthetics, in that the temporary decrease followed by an increase which is found in animals does not occur in plants. If this should prove to be generally true, it is significant.

The facts developed in these studies are of considerable interest but it seems wiser to defer their interpretation until more information is available. They offer suggestions for further attacks upon the problem and have an important bearing on the theory of anesthesia. These investigations are being continued and it is expected that additional results will be forthcoming in the near future.

It may be added that throughout these articles the term respiration is used as meaning oxidative processes which furnish energy, beginning with the taking up of O_2 and ending with the production of

CO_2 and H_2O. The consumption of O_2 and the production of CO_2 furnish the most convenient means of measuring respiration and it is always desirable to study both together. If we find that O_2 is consumed but no CO_2 is produced, as in some of Warburg's experiments,[15] we may speak of respiration. If on the other hand CO_2 is produced but no free O_2 is consumed, as is the case in anaerobic respiration, we may regard this as respiration also. Ordinarily a rise in the rate of CO_2 output is interpreted as a rise in the rate of respiration, but it might happen that the sudden production of an acid (*e.g.* lactic acid) might set free CO_2 from carbonates already present in the tissue. When there is reason to suspect that this is the case special precautions must be taken to ascertain how the CO_2 is produced.

Some writers endeavor to make a distinction between oxidation in the living cell and that which occurs after death. In the opinion of the writer there is no valid ground for such a distinction, and the term respiration is here used to include processes which occur immediately after death.

SUMMARY.

A series of investigations on respiration with improved quantitative methods has been commenced. The first of these are here described. They show that when anesthetics are employed in sufficient concentration to produce any result, plants show a rise in the rate of respiration which is followed by a fall. In the animals studied, the rise (found in higher concentrations only) was preceded by a temporary fall which is not entirely due to lowering of muscular activity or tonus. In lower concentrations the effect on animals was merely a decrease of respiration.

The results of all the investigations are opposed to the theory of Verworn.

[15] Warburg, *Ergebn. Physiol.*, 1914, xiv, 319.

COMPARATIVE STUDIES ON RESPIRATION.

II. The Effect of Anesthetics and Other Substances on the Respiration of Aspergillus niger.

By F. G. GUSTAFSON.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, September 6, 1918.)

The object of this investigation is to compare the action of anesthetics and some other substances on the respiration of a fungus with similar effects in other groups of organisms.

The fungus which has been used throughout these experiments is *Aspergillus niger*. This was selected because it grows well in a nutrient solution and forms on the surface of the liquid a compact mass of hyphæ, which is easily handled. *Penicillium* sp. was also tried, but this forms loose tufts of hyphæ, which are very hard to manipulate.

The fungus was grown in a nutrient solution of 40 gm. of cane sugar, 2 gm. of KNO_3, 1 gm. of KH_2PO_4, and 0.5 gm. of $MgSO_4$ in 800 cc. of tap water. This solution has an acid reaction, a fact which may have been beneficial in preventing the growth of bacteria. The cultures were examined at various times for bacteria but at no time were any found. The fungus was grown from spores in 100 cc. Erlenmeyer flasks, in about 30 cc. of sterilized nutrient solution. They were kept slightly above room temperature. Spores were usually formed in 3 or 4 days after inoculation, so it was found best to use the cultures when 2 days old. The respiration was at its maximum at this time. If older cultures were taken respiration was not so great.

The indicator method[1] was used for determining the amount of CO_2 given off. For buffer solutions borax and boric acid solutions were used in various mixtures. A table for making up buffers of

[1] Haas, A. R., *Science*, 1916, xliv, 105.

any desired pH value by means of borates and boric acid has been published by Palitzsch[2] and later republished by Osterhout and Haas.[3]

In the experiments 10 cc. of tap water were used in each tube to which five drops of a 0.01 per cent solution of phenolsulfonephthalein were added as indicator. That this indicator was not toxic to the fungus was proved by sowing spores in a nutrient solution containing the same concentration of indicator as used in the experiments. A control with the same amount of spores was inoculated at the same time and grown under similar conditions. The two cultures matured at the same time and behaved similarly when treated with anesthetic.

Respiration was measured in the nutrient solution and in sugar solutions in preliminary experiments, but as the results were the same as when tap water was used, the nutrient solution was replaced by tap water, which was easier to handle. The tap water was very near the neutral point and was brought to the desired alkalinity by boiling off some of the dissolved CO_2 or by washing out some of the CO_2 by means of a current of air free from CO_2.

When an experiment was started, some of the fungus was thoroughly rinsed in tap water to wash off the nutrient solution. Any adhering bubbles of gas enclosed in the interwoven hyphæ had to be got rid of before the readings were begun, either by gently squeezing the material between the fingers or by a continued shaking in several changes of water. The mass of hyphæ was then separated into pieces small enough to be shaken about easily in the tube. They were then put into a Pyrex glass tube which was closed at one end, while at the other was attached a paraffined rubber tube about 3 inches long.[1] Before putting the fungus in the tube, 10 cc. of tap water (plus five drops of indicator) had been poured in. When the fungus had been put in, the rubber tube was closed with a spring clamp, in such a way as to enclose a small bubble of air, to act as a stirrer. In all experiments this bubble was made as nearly the same size as possible. The tube was now shaken until any gas that may have adhered to the material was detached. The color of the experi-

[2] Palitzsch, S., *Biochem. Z.*, 1915, lxx, 333.

[3] Osterhout, W. J. V., and Haas, A. R. C., *Science*, 1918, xlvii, 420.

mental tube was then matched with that of a buffer tube of the same size (and having the same concentration of indicator), using a constant source of light ("Daylight" lamp).[4]

The alkalinity of the water was brought (by the means mentioned above) to a point a little above pH 7.60; after adding the material it was allowed to stand until it fell to pH 7.60, which was taken as the starting point in all experiments. The time was noted and the tube put aside for a minute or two, when it was gently shaken to distribute the CO_2 throughout the solution. The tube was again matched, this time with another buffer tube having a pH value of 7.25. If the experimental tube had not yet reached this, it was repeatedly examined at very short intervals until the two matched. The change in pH was from 7.60 to 7.25, which was the standard unit of measurement in all cases.

In starting an experiment the time required for this change was noted and the material was rejected unless this time was practically constant for at least three periods before any anesthetic was added. The time varied with the amount of material used, but was made as near to 3 minutes as possible in each experiment. When the endpoint was reached, the material was taken out and rinsed in tap water before starting a new measurement with a new solution.

When a practically constant rate of respiration had been obtained, the material was placed in a solution of the desired concentration of the anesthetic. After the addition of the anesthetic the respiration was measured in the same manner as before. The experiments with anesthetics lasted from half an hour to 2 hours.

On account of the short periods used it was found impossible to carry on a control simultaneously with the experiment. Therefore a large number of control experiments were made from a number of cultures and at different times during the period of investigation. These control experiments agreed very closely, so they were averaged to make up a control curve, which has been used in all figures. As will be noticed from the figures, the control shows a gradual decrease in rate of respiration. This is probably due to a decrease in oxidizable

[4] For other details see the introductory article by Osterhout, W. J. V., *J. Gen. Physiol.*, 1918, i, 171.

material within the cells, as this decrease did not occur when a 3 per cent sugar solution was used in place of tap water.

All the experiments were made at room temperature (18–20°C.).

In regard to the accuracy of the results it may be stated that in no case did the probable error exceed 2.4 per cent of the mean.

The measurement of respiration at frequent intervals, as here practised, has great advantages. Methods heretofore employed in measuring respiration have usually necessitated the use of long periods. It will be noticed readily from curves here presented that if periods of an hour's duration had been used no increase would have been found except in one or two cases, because the increase, though distinct enough, lasts but a short time (in many experiments only from 5 to 10 minutes). This increase is followed by a decrease, and if the total amount of CO_2 given off in 1 hour were measured, the large output for a short time would be more than counterbalanced by the longer period of small output, so that the total would be below normal, and we should record a decrease instead of an initial increase followed by a decrease. In the writer's opinion there is no doubt that this is what has happened in many cases, where only a falling off of respiration has been reported. Schroeder[5] got a decrease in respiration of *Aspergillus niger* when he used 6 and 7 per cent ether. In the present investigation 3.65 and 7.3 per cent ether both gave a decided increase (except in a few cases), but this was followed by a drop, and if 1 or 2 hour periods, as Schroeder used, had been employed, it is very likely that only a decrease would have been noticed. Kosinski[6] as the result of experiments with *Aspergillus niger* reports an increase in CO_2 output with 0.5 per cent ether and a decrease with 5 and 7 per cent; with 0.2 per cent cocaine and 0.02 per cent strychnine nitrate an increase was observed.

The first experiments were made with formaldehyde. A few experiments were conducted at first with 0.2 per cent (by volume), but as this concentration gave a very small change, 0.4 per cent was used. This stronger concentration gave a very large increase followed by an abrupt decrease. The results are shown in Fig. 1. Six experiments

[5] Schroeder, H., *Jahrb. wiss. Bot.*, 1907, xliv, 409.

[6] Kosinski, I., *Jahrb. wiss. Bot.*, 1902, xxxvii, 163.

were made with this concentration. Their average happens to correspond almost exactly with one experiment, and this experiment

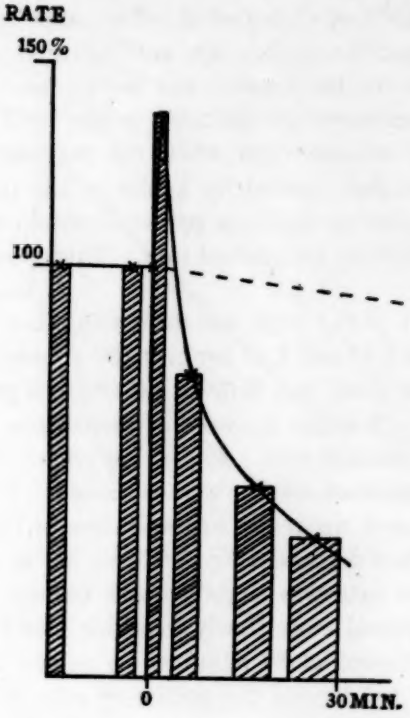

FIG. 1. Curve showing the effect of 0.4 per cent formaldehyde on the respiration of *Aspergillus niger*. The shaded portions represent the periods during which respiration was measured. The unshaded portions represent the intervening time during which no measurement was made (*e.g.*, time spent in changing the material from one solution to another). The horizontal part of the curve shows the normal respiration; the remainder the respiration in 0.4 per cent formaldehyde. The dotted line represents the respiration of a control in tap water. Time is reckoned from the beginning of the exposure to formaldehyde. The normal rate (which is taken as 100 per cent) corresponds to a change from pH 7.60 to pH 7.25 in 2.75 minutes.

was used instead of the average in drawing the curve, as will be seen in Fig. 1. The periods during which the measurements were made are indicated by shading. The rate which is obtained for each period

is an average for that period, and if the rate is changing, it is evident that this average rate will in general occur somewhere near the middle of the period. Hence in drawing the curve the points are taken in the middle of each period. The other curves are made in the same way but the periods are not indicated.

After exposure to the reagent has begun, the intervals between periods of measurement are included in the total time of exposure to the reagent, because even when the material is momentarily lifted out, it remains covered by a film of the reagent. The relative rate of respiration for each period is obtained by dividing the rate for that period by the normal rate. This applies to all the subsequent work.

The main part of the work was done with ether. Various concentrations between 0.37 and 1.46 per cent (by volume) were tried in the beginning but as these had little or no effect, higher concentrations were employed. A slight increase of respiration, which lasted for some time, was obtained with 1.46 per cent ether. With 3.65 per cent three distinct types of results were obtained. The first and most common type was a sharp rise in respiration in the first period, followed by a gradual decrease (Fig. 2, Curve A), so that at the end of half an hour the rate was slightly below normal. From this point on the rate decreased more slowly, reaching about 60 per cent of the initial rate at the end of 80 minutes. A second type gave a curve with a flattened top having the maximum rate of respiration in the third or fourth period. Only very few experiments showed this type. The third type never gave a rise, but always a slow decrease from the beginning (Fig. 2, Curve B).

These three types were constant in the sense that each culture always gave only one result, no matter how many experiments were made. Though the cultures were grown under identical conditions, yet this difference in behavior was always noted.

A saturated solution of ether was also tried. As this is approximately 7.3 per cent by volume, the latter designation has been used for the sake of convenience. With 7.3 per cent only one type of result was noticed, even when the same cultures were used with which different results had been obtained with 3.65 per cent. A very pronounced increase was noticed in the first period followed by an almost equally

rapid decrease, so that in from 6 to 10 minutes the rate was back at normal. At the end of an hour respiration was nearly at a standstill (Fig. 2, Curve C).

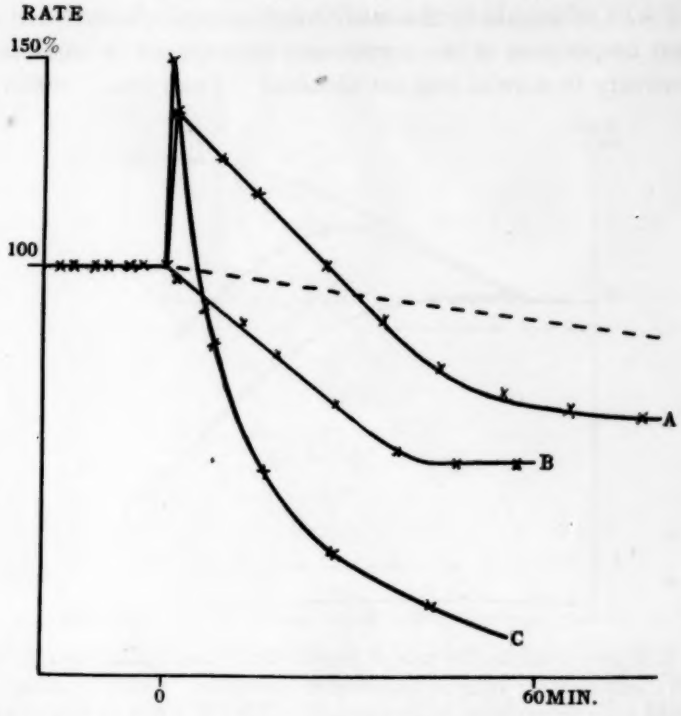

FIG. 2. Curves showing the effect of ether on the respiration of *Aspergillus niger*. Curve A, in 3.65 per cent ether; Curve B, a different culture in 3.65 per cent ether; Curve C, in 7.3 per cent ether. The horizontal part of the curves shows respiration in tap water before exposure to ether. Dotted line shows respiration of a control in tap water. The normal rate (which is taken as 100 per cent) corresponds to a change from pH 7.60 to pH 7.25 in 3.25 minutes for Curve A and in 3 minutes for Curves B and C. Curve A represents the average of four experiments; Curves B and C, the average of five experiments. Probable error, less than 2.4 per cent of the mean.

The results with ether are shown in a different manner in Fig. 3.

A number of experiments on recovery were conducted with 7.3 per cent ether. The result of these experiments was, that if respiration

had risen and fallen again to normal and the material was then taken out of the ether solution and put in a nutrient solution, it was found on observing it 2 to 3 hours later that respiration was practically normal (Fig. 4, Curve B). If on the other hand the mater al was allowed to remain in the ether solution until the rate had fallen to about 60 per cent of the normal and then placed in nutrient solution recovery to normal was not obtained. From this it would seem

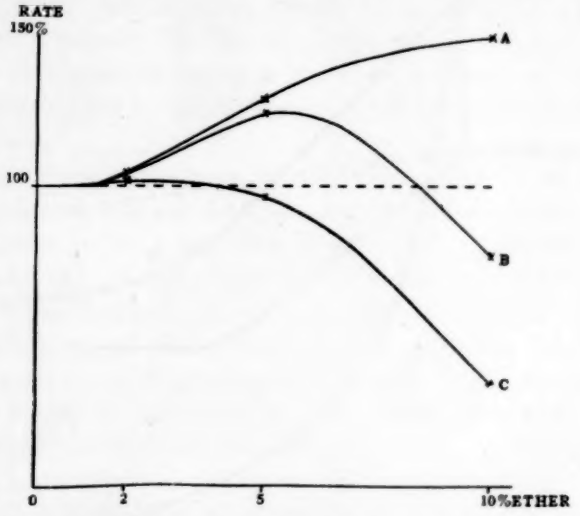

FIG. 3. Curve A shows the rate of respiration of *Aspergillus niger* in various concentrations of ether after an exposure of 2 minutes. Curve B shows rate of respiration after an exposure of 10 minutes. Curve C shows rate of respiration after an exposure of 30 minutes. The dotted line shows the normal respiration. The figures expressing the per cent of ether should be corrected by multiplying by 0.73.

that only the increase in respiration is reversible and that when a decrease takes place an irreversible reaction involving injury is going on.

A 20 per cent solution (by volume) of acetone was also employed. A very large increase took place, with the maximum during the second period (Fig. 5, Curve B). This concentration of acetone was very much less toxic than 7.3 per cent ether.

Alkaloids usually have a special effect. For this reason it was thought best to try caffeine as a representative of this group. Low

concentrations, such as Haas[7] reports using, had no effect whatsoever. The lowest concentration that showed any effect was 0.5 per cent (Fig. 5, Curve A). This gave a decrease in respiration from the beginning. In the first two periods there was a decrease of nearly 20 per cent below normal; then followed several periods with hardly any decrease at all. Several experiments with a saturated solution

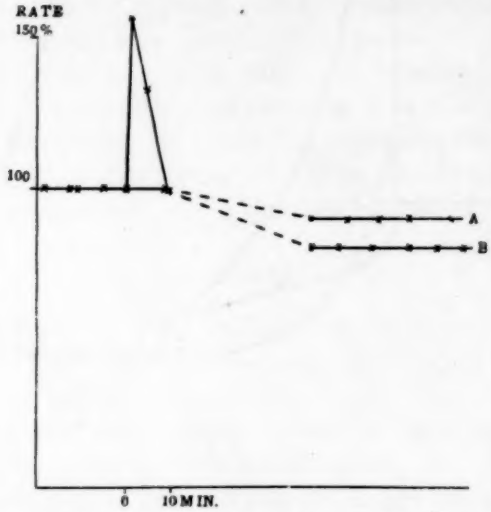

FIG. 4. Curve B shows the respiration of *Aspergillus niger*, first for 20 minutes in tap water (horizontal unbroken line), then for 9.5 minutes in 7.3 per cent ether, then for 130 minutes (interval shortened in figure to save space and denoted by dotted lines) in nutrient solution, and finally for 36 minutes in tap water (unbroken line). Curve A shows the respiration of a control placed in tap water for 30 minutes, then for 130 minutes in nutrient solution (dotted line), then for 34 minutes in tap water (unbroken line). The normal rate (which is taken as 100 per cent) corresponds to a change from pH 7.60 to pH 7.25 in 3 minutes. Average of three experiments. Probable error less than 2.4 per cent of the mean.

of caffeine were also performed. An initial increase amounting to about 15 per cent above normal was noticed (Fig. 5, Curve C). This was followed by a gradual decrease till a rate of 60 per cent of the normal was reached, when the rate of respiration became stationary and remained so till the end of the experiment (over half an hour).

[7] Haas, *Science*, 1917, xlvi, 462.

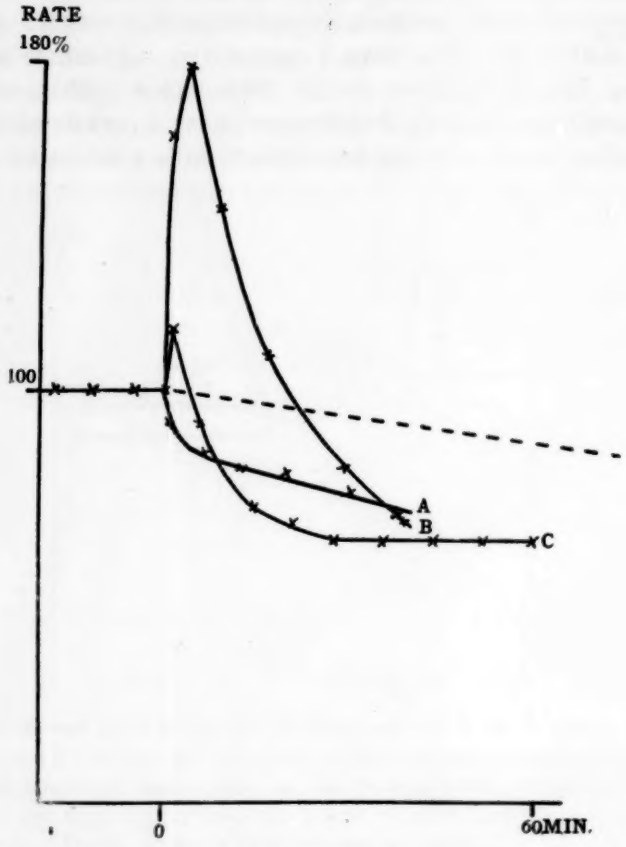

FIG. 5. Curve B shows the respiration of *Aspergillus niger*, first for 20 minutes in tap water (horizontal part of curve), then in 20 per cent acetone. Curve A shows the respiration, first in tap water (horizontal part of curve), then in 0.5 per cent solution of caffeine. Curve C shows respiration, first in tap water (horizontal part of curve), then in saturated solution of caffeine. The dotted line shows the respiration of a control in tap water.

The normal rate (which is taken as 100 per cent) corresponds to a change from pH 7.60 to pH 7.25 in 2.75 minutes for Curve A; in 3.25 minutes for Curve B, and in 3 minutes for Curve C.

Curve A represents the average of seven, Curve B of four, and Curve C of two experiments. Probable error less than 2.4 per cent of the mean.

The action of caffeine seems to be very much slower and less pronounced than that of the other substances tried.

The absence of any noticeable buffer action in any of the solutions was determined by means of an apparatus which has been described by Osterhout.[8] A measured amount of CO_2 is introduced into a certain amount of solution with indicator and the change in pH is noted. This is compared with the change caused by an equal amount of CO_2 in the same amount of tap water.

The above results agree in the main with those obtained by Haas[7] on *Laminaria*. Usually stronger solutions were needed to cause any effect on the respiration of *Aspergillus niger*, and the changes were not so great. With all reagents tried there was an increase, though with some concentrations only a decrease was noted.

SUMMARY.

1. In concentrations which are high enough to produce any effect, formaldehyde, ether, and acetone cause an increase, followed by a decrease, in the rate of respiration.

2. 3.65 per cent ether, which causes an increase with certain cultures, produces only a decrease with others.

3. The reaction producing an increase in the respiration with 7.3 per cent ether is a reversible process, while the reaction producing the decrease is not reversible.

4. 0.5 per cent caffeine produces only a decrease in respiration while a saturated solution causes an increase, which is followed by a decrease.

[8] Osterhout, *J. Biol. Chem.*, 1918, xxxv, 237.

COMPARATIVE STUDIES ON RESPIRATION.

III. The Effect of Ether on the Respiration and Growth of Bacillus subtilis.

By MATILDA MOLDENHAUER BROOKS.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, September 6, 1918.)

Although so much stress is laid on the physiological reactions of bacteria in their identification and classification, little investigation (except to distinguish aerobic from anaerobic forms) has been devoted to the fundamental process of respiration in these organisms. In order to throw some light on the nature of this process and to compare the results with those obtained with other plants and with animals, a series of studies upon the respiration of bacteria has been undertaken. The present paper describes the results of some experiments on the effects of ether.

The organism selected for experimentation was *Bacillus subtilis*, isolated from water and inoculated upon agar-agar daily during the month previous to experimentation, so that a pedigreed culture was at hand. Several other organisms were tried; namely, *Bacillus mycoides*, *Staphylococcus pyogenes albus*, *Staphylococcus citreus*, *Bacillus typhosus;* but *Bacillus subtilis* was preferred because it was easier to handle. Bacteria were incubated for 16 to 18 hours at 37°C. and were then washed into a test-tube with a few cc. of tap water containing indicator. A thick emulsion was made by repeatedly drawing the liquid into, and ejecting it from a pipette which was drawn out to a very fine point.

The respiration of the bacteria was tested in boiled tap water. The boiling rendered the water alkaline by driving off the CO_2. To each 2 cc. of solution was added one drop of a 0.01 per cent aqueous solution of phenolsulfonephthalein. The color of the solution was compared with the colors of a set of standard buffer solutions, made according to Sörensen's directions with KH_2PO_4 and Na_2HPO_4, hav-

ing the same concentration of phenolsulfonephthalein as the experimental solution and contained in Pyrex tubes of the same size. The tap water was found to have a pH value of 8.3.

All experiments were done in Pyrex tubes each containing a total of 5 cc. of liquid as follows: Various amounts of tap water plus three standard drops of bacterial emulsion and sufficient saturated solution of ether in tap water to make the following concentrations of ether by volume: 0.037, 0.183, 0.329, 0.438, 0.95, 1.46, 2.9, 4.38, 5.84, and 7.3 per cent.

Each tube, therefore, contained a total volume of liquid amounting to 5 cc. in which the concentration of indicator was the same. The number of bacteria was made as nearly equal as possible in the different tubes by taking a uniform emulsion, mixing it thoroughly before taking the drops from it, and using a standard dropper so as to have the drops of equal size. The emulsion was transferred almost simultaneously to all the tubes. The tubes were then quickly closed (by clean corks boiled in paraffin) with the exclusion of all air and determinations were made by comparison with the standard set of buffer solutions contained in tubes of the same size. In every case there was a control consisting of organisms in tap water without ether, and of ether in tap water without organisms.

To see if phenolsulfonephthalein is toxic to bacteria, several trials were made by placing bacteria in a liquid of known pH value and after respiration had gone on for a definite time adding the indicator, and comparing that result with the color of a control tube to which the indicator was added at the beginning of the experiment. No difference in rate of respiration was observed.[1]

It was also found that ether has no buffer action to interfere with the measurement of the rate of respiration.

That the bacteria under these conditions give off no alkali or acid (other than carbonic) is shown by driving off the CO_2 at the end of the experiment. The solution then returns to the original pH value.

A great deal of variation in cultures of different ages was observed. Those more than 24 hours old have a markedly lower rate

[1] For other details see Paper I of this series (Osterhout, W. J. V., *J. Gen. Physiol.*, 1918, i, 171.

of respiration than cultures between 18 and 24 hours old. The time required to change the pH value from 8.3 to 8.1 by respiration may be only a few minutes when the culture is young, while as much as 6 hours may be necessary in the case of an older culture. This might be due to the fact that in the older culture the bacteria have gone into the resting stage or have produced spores. In this stage ether has little or no action upon the respiration of the bacteria.

The temperature maintained was from 18–20°C. as this was found to be sufficiently constant. Presumably the respiration of bacteria has the ordinary temperature coefficient of 2. A few experiments were tried at about 1°C. and it was found that exposure to this temperature for 15 minutes had a marked after effect, as little or no respiration was observed for about 6 hours.

In the earlier experiments it was thought desirable to permit respiration to produce a definite change in pH value and then wash out the CO_2 with H_2 or with air free from CO_2. But this was found to injure the bacteria (owing probably to mechanical or chemical disturbances), so that the rate of respiration was greatly diminished. Therefore it was necessary to use separate tubes for the control and for each concentration of ether (instead of finding the normal rate of respiration of a given tube and afterward exposing it to ether). It was possible to do this as a number of readings showed a fairly constant rate of respiration.

The rate is obtained by taking the reciprocal of the time required to produce a given change in pH value. It is expressed as per cent of the normal rate which is always taken as 100 per cent.

Fig. 1 shows a comparison between the respiration of *Bacillus subtilis* in tap water and in four concentrations of ether (by volume). In every case the rate is more rapid at first and becomes slower as the reaction of the medium becomes more acid. The normal curve, indicated by the dotted line, shows a slower rate than any of the other curves. In no case does the indicator show the pH value to be below 6.8 to 6.4.

When higher concentrations are used, the respiration is more rapid during the first few pH intervals and much slower during the last few. In fact, concentrations of ether as high as 1.46 per cent and upward never reach so low a pH value as concentrations below 1.46 per

cent. For example, the curve readily shows that 7.3 per cent ether, although causing a rapid respiration at first, does not produce a lower pH value than 7.9. After allowing these tubes to stand several days, they change about 0.2 pH unit, or reach 7.7 pH, owing probably to the fact that there are a few surviving organisms that are perhaps more resistant than the majority, as later experiments indicate.

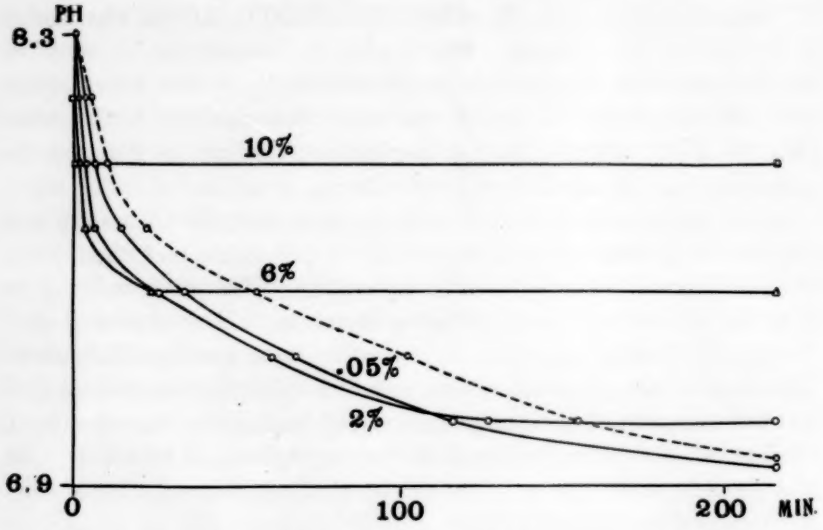

FIG. 1. Curves showing the effects of various concentrations of ether on the respiration of *B. subtilis*. Dotted line, control. Average of two experiments. The figures expressing the per cent of ether should be corrected by multiplying by 0.73.

Fig. 2 shows more readily the effect of 7.3 per cent ether upon the rate of respiration as compared with the normal rate (dotted line). There is a sudden outpouring of CO_2 followed by cessation of respiration.

Fig. 3 plotted in the same way, shows the effect of lower concentrations of ether upon the rate of respiration as compared with the normal curve (dotted line).

Curve 1 in Fig. 4 shows the rate of respiration in various concentrations of ether during the first interval in which the pH value changes

from 8.3 to 8.1. The rate is taken as the reciprocal of the time required to change the pH value from 8.3 to 8.1. The curve shows a gradual increase in respiration as the concentration of ether increases. The curve rises more rapidly from 2.9 to 5.84 per cent, and very rapidly near 7.3 per cent. In fact, the change in pH value at 7.3 per cent is almost instantaneous.

FIG. 2. Curves showing the effect of 7.3 per cent ether on the rate of respiration of *B. subtilis*. Dotted line, control. The normal rate (which is taken as 100 per cent) corresponds to a change from pH 8.3 to pH 8.1 in 7 minutes. Average of three experiments.

For the sake of comparison, a similar series of observations was made with the same concentrations of ether dissolved in 0.85 per cent NaCl. Curve 2 of Fig. 4 shows increase in respiration with increase in concentration; the curve rises rapidly at first, then more slowly and finally quite steeply. No sudden outpouring of CO_2 is observed in 7.3 per cent ether as is the case in the tap water (Curve 1). This seems to indicate an antagonism between the action of NaCl and ether.

The question arises whether the sudden outpouring of CO_2 in 7.3 per cent ether is due to the sudden production of a great excess of CO_2 or merely to the sudden liberation of CO_2 previously stored up in the cells (either as CO_2 or in the form of carbonates and bicarbon-

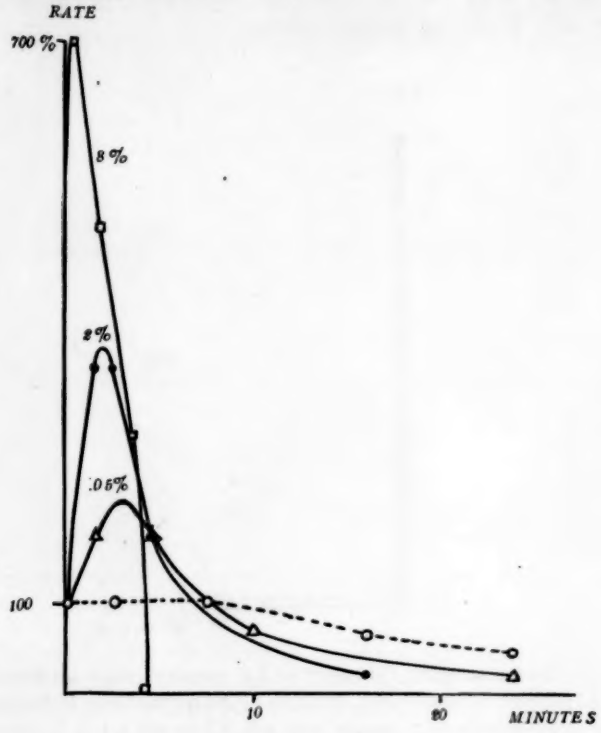

FIG. 3. Curves showing the effect of ether on the rate of respiration of *B. subtilis*. Dotted line, control. The normal rate (which is taken as 100 per cent) corresponds to a change from pH 8.3 to pH 8.1 in 5 minutes. Average of two experiments.

The figures expressing the per cent of ether should be corrected by multiplying by 0.73.

ates). It seems more probable that the latter is the case but there is also a sudden increase in O_2 consumption when the bacteria are placed in 7.3 per cent ether, as is shown by using *Limulus* blood as an indicator.[2]

[2] Osterhout, *J. Gen. Physiol.*, 1918, i, 167.

The great increase in respiration in 7.3 per cent ether in tap water raises the question whether the bacteria were injured. To obtain some light on this question, the contents of each tube were tested (after an exposure to ether lasting 20 minutes) by plating the bacteria on Petri plates and counting the colonies. Two loopfuls of solution from each Pyrex tube were diluted in 10 cc. of sterile water and from

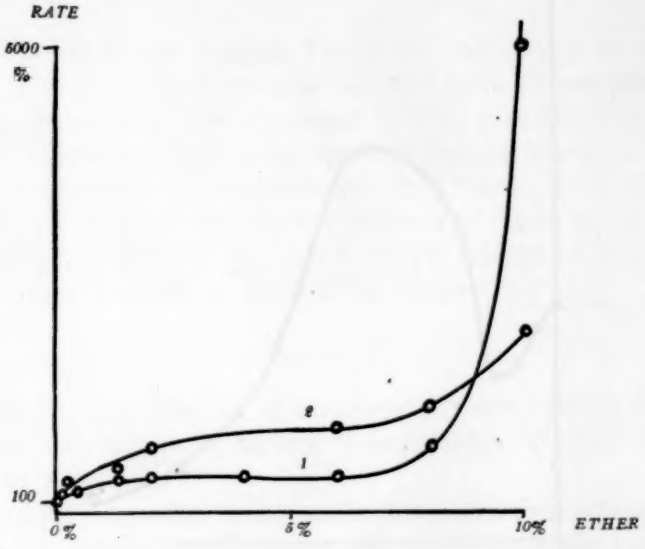

FIG. 4. Curves showing the effect of various concentrations of ether on the rate of respiration of *B. subtilis* in tap water (Curve 1) and in 0.85 per cent NaCl (Curve 2). The normal rate (which is taken as 100 per cent) corresponds to a change from pH 8.3 to 8.1 in 5 minutes. Average of three experiments.

The figures expressing the per cent of ether should be corrected by multiplying by 0.73.

these, one loopful was placed in 10 cc. of agar-agar and plates were poured in triplicate. These plates were incubated over night at 37°C. and counted the next day. The results were expressed as per cents of the normal. These per cents are plotted as ordinates in Fig. 5 and the concentrations of ether as abscissæ. In performing this experiment all precautions were observed to ensure sterility of the media.

To show what effect a longer exposure to ether would have upon the bacteria, another set of plates was poured in the same manner as the first set except that the bacteria were allowed to remain in ether for 1 hour. The solutions tried were (1) tap water, (2) saturated solution of ether (about 7.3 per cent in tap water), (3) 0.85 per cent

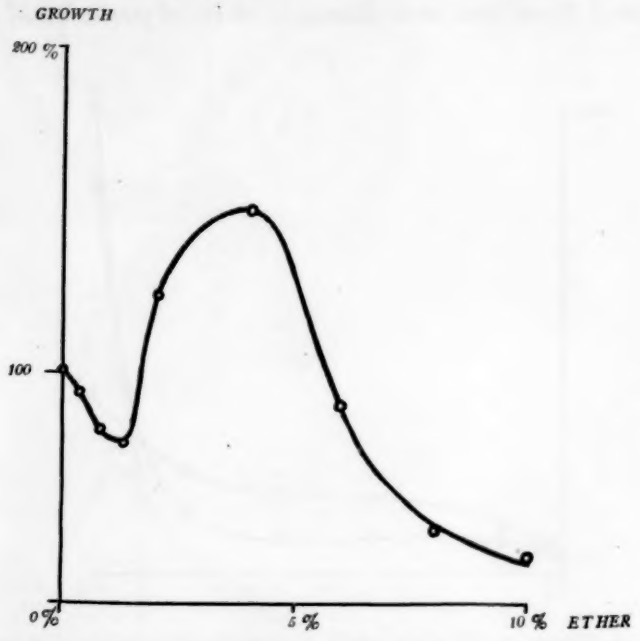

FIG. 5. Curve showing the effect of various concentrations of ether on the growth of *B. subtilis*. The ordinates denote the number of colonies expressed as per cent of the normal, which is taken as 100 per cent.

The figures expressing the per cent of ether should be corrected by multiplying by 0.73.

NaCl in tap water, and (4) saturated solution of ether in 0.85 per cent NaCl solution. The following results were obtained, averaging nine trials and counting tap water as 100 per cent.

Tap water.	Ether in tap water.	0.85 per cent NaCl.	Ether in 0.85 per cent NaCl.
per cent	*per cent*	*per cent*	*per cent*
100	10	112	14

Fig. 5 shows that as the concentration of ether increases up to 1.1 per cent there is a decrease in rate of growth, reaching a minimum at about 0.95 per cent. Concentrations of ether from 1.1 to 4 per cent produce increase in growth with a maximum at 2.9 per cent. All higher concentrations of ether produce decrease in growth. These observations show that ether is toxic in low concentrations and in very high concentrations, while intermediate concentrations stimulate growth.

A possible correlation between Fig. 4 and Fig. 5 may be made. In Fig. 4 there is a steeper ascent in the curve up to 1.1 per cent concentration of ether, and this corresponds to the first period of toxicity in Fig. 5. Then there follows a more uniform increase in respiration in Fig. 4 corresponding to an increase in growth in Fig. 5. Above 2.9 per cent ether, Fig. 4 shows the very steep ascent of the curve (or very rapid respiration) and Fig. 5 shows a sudden descent of the curve with a decrease in growth or death of the bacteria.

SUMMARY.

1. In all the concentrations of ether studied (from 0.037 to 7.3 per cent) there is an increase in the rate of respiration of *Bacillus subtilis* followed by a decrease.

2. In 7.3 per cent ether in tap water there is an extraordinary increase in the output of CO_2 (amounting to 50 times the normal). This does not occur when 0.85 per cent NaCl is added, which indicates antagonism between ether and NaCl.

3. Ether is toxic in low concentrations (0.037 to 1.1 per cent) and high concentrations (3.65 to 7.3 per cent) but in intermediate concentrations (1.1 to 3.65 per cent) stimulates growth.

COMPARATIVE STUDIES ON RESPIRATION.

IV. The Effect of Ether on the Respiration of Wheat.

By HELEN STILLWELL THOMAS.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, September 6, 1918.)

Investigations on the effect of anesthetics on the production of CO_2 in higher plants have yielded somewhat contradictory results. Appleman[1] found that the respiration of potatoes exposed to ethyl bromide vapor for half an hour was approximately doubled. Irving[2] noted that the respiration of barley shoots was first increased and then decreased by chloroform vapor. Laurén[3] stated that some seedlings showed an increase in respiration when exposed to ether or chloroform, provided the treatment was not carried far enough to injure the plant permanently; other seedlings showed a decrease in respiration. Elfving[4] also observed an increase in respiration with ether and chloroform. Ewart[5] found that chloroform increased the respiratory activity in *Elodea*.

On the other hand, no increase in respiration was observed by Detmer,[6] nor by Bonnier and Mangin.[7] Tashiro[8] states that the respiration of dry seeds under the influence of anesthetics was decreased.

In no case where only a decrease was found was the time curve determined, and it is therefore impossible to say whether there was a preliminary increase. In Irving's experiments, where time curves

[1] Appleman, C. O., *Am. J. Bot.*, 1916, iii, 223.

[2] Irving, A. A., *Ann. Bot.*, 1911, xxv, 1077.

[3] Laurén, W., Über den Einfluss von Ätherdämpfen auf die Atmung von Keimlingen, Dissertation, Helsingfors, 1891 (*Bot. Jahresber.*, 1892, xx, 92).

[4] Elfving, *Oefversigt Finska Vetensk. Soc. Forh.*, 1886, xxviii.

[5] Ewart, A. J., *Ann. Bot.*, 1898, xii, 415.

[6] Detmer, W., *Landw. Jahrb.*, 1882, xi, 227.

[7] Bonnier, G., and Mangin, L., *Ann. sc. nat.*, 1886, cxi, series 7, 16.

[8] Tashiro, S., *Am. J. Physiol.*, 1913–14, xxxii, 107.

were determined, the periods were usually comparatively long, 1 or 2 hours, and therefore the complete series of changes in the rate of respiration could not be followed.

The material chosen for these experiments was wheat, as representing the flowering plants. The anesthetic used was ether in 7.3 and 3.65 per cent solutions, by volume.

Wheat seeds were soaked for 12 hours, during which time the process of germination enters upon its first stages. Seeds were selected which were as nearly alike as possible; twenty-five were placed in a Pyrex tube with 10 cc. of water containing five drops of the indicator, 0.01 per cent phenolsulfonephthalein. The tube was closed so that all air was excluded and the seeds themselves acted as a stirrer.

It was found convenient to allow the seeds to give off enough CO_2 to change the solution from a pH value of 7.78 to a pH value of 7.36. This range of color change was arbitrarily selected as definite and easy to read, and formed the standard for all the experiments. Tap water which had been boiled to drive off the CO_2 sufficiently to give a pH value slightly higher than 7.78 was used in all cases. At the end of each period the liquid was poured out and fresh tap water was added. A normal rate of respiration was first established by repeated determinations,[9] then the reagent was added and the rate again determined. All results were expressed in per cent of the normal rate, which was called 100 per cent.

In these experiments the colors were matched with the aid of a "Daylight" lamp, which gave a constant source of light. The experiments were made at room temperature, about 18–20°C.[10]

The stock solution of ether was a saturated solution in tap water (containing the indicator of the standard concentration). In order to have the solution of the right pH value at the start (pH 7.78), two bottles of 7.3 per cent ether were kept on hand;[11] these had different pH values and by mixing, the desired value was attained.

[9] This was taken as the reciprocal of the time required to change the pH value from 7.78 to 7.36.

[10] For other details of technique see Paper I (Osterhout, W. J. V., *J. Gen. Physiol.*, 1918, i, 171.

[11] Glass stoppered bottles of Kavalier glass (which does not give off sufficient alkali to interfere with the experiment) were employed for this purpose.

In 7.3 per cent ether solutions there was an immediate increase in the rate of respiration which reached a maximum of 206 per cent (106 per cent increase) in 8 to 9 minutes (Fig. 1, Curve A). This

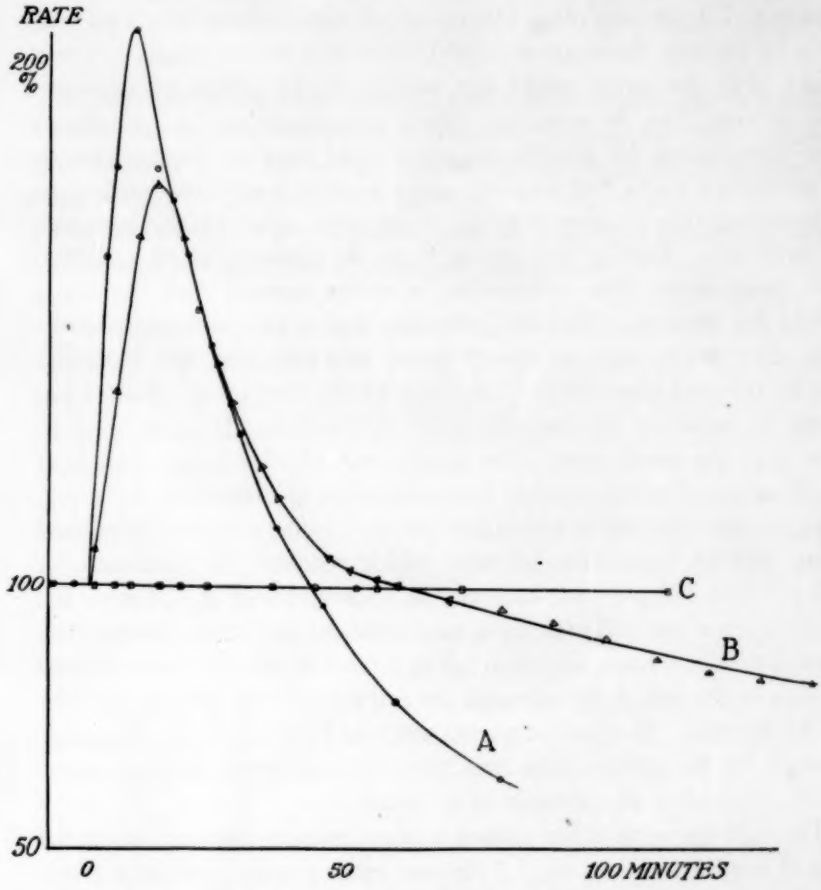

FIG. 1. Curves showing the rate of respiration of wheat seeds in 7.3 per cent ether solution (Curve A); in 3.65 per cent ether (Curve B); and in tap water (Curve C). Time is reckoned from the beginning of exposure to the ether solutions. The normal rate (which is taken as 100 per cent) corresponds to a change from pH 7.78 to pH 7.36 in 1.93 minutes for Curve A, in 2.28 minutes for Curve B, and in 1.53 minutes for Curve C. Curve A represents the average of ten, Curve B of five, and Curve C of four experiments. Probable error is less than 5 per cent of the mean.

was followed by a more gradual fall which reached the normal rate in 43 minutes and continued to fall, so that at the end of 90 minutes the rate was only 60 per cent of the normal.

Some experiments were made to determine the recovery after exposure to 7.3 per cent ether for periods of various lengths. This was done by putting the seeds in large Petri dishes to germinate. It was found that the seeds would not recover if the period of exposure was greater than 30 minutes. Short exposures (up to 8 minutes) were stimulating, for growth was more rapid than the normal growth of unexposed seeds, but any exposures beyond 8 minutes resulted in a decreased rate of growth up to 30 minutes, after which there was no recovery. During the period from 30 minutes to 43 minutes, the curve shows that respiration is above normal, but the seeds would not recover. This was probably due in part to rapid respiration after death, such as Haas[12] found in *Laminaria*, but it should not be inferred that death took place at the end of 35 minutes because no recovery was possible after this period. It is more probable that the seeds were alive at the end of 35 minutes and that death occurred after removal from the ether solution.

In the experiments in which 3.65 per cent ether solutions were used there was an immediate increase which reached the maximum of 178 per cent (78 per cent increase) in 13 minutes (Fig. 1, Curve B). This increase was followed by a more gradual fall which reached the normal rate in 1 hour, and then fell still more gradually below normal so that at the end of 140 minutes the respiration was only 81 per cent of the normal. In all experiments with 3.65 per cent ether the seeds showed by the germination test that the anesthetic did not cause death, even after an exposure of 12 hours.

The 3.65 per cent ether caused a more gradual rise and fall in the rate of respiration than the 7.3 per cent ether and the increase was not so great. In both cases, however, there was a definite rise followed by a fall.

In 0.73 per cent ether the rise was less than in 3.65 per cent and recovery was possible even after an exposure of 12 hours.

[12] Haas, A. R. C., *Proc. Nat. Acad. Sc.*, 1917, iii, 688.

The consumption of oxygen was determined by Winkler's method.[13] During an exposure of 30 minutes to 7.3 per cent ether the consumption of oxygen was 145 per cent of the normal (average of four experiments) while the production of CO_2 was 165 per cent of the normal.

SUMMARY.

These experiments show that 7.3 and 3.65 per cent ether solutions cause an increase in respiration followed by a decrease. The results agree with those of Haas[14] on *Laminaria*, of Gustafson[15] on higher fungi, and of Mrs. Brooks[16] on bacteria.

They do not agree with the theory of Verworn that anesthesia is a kind of asphyxia and that it decreases respiration.

[13] Cf. Osterhout, W. J. V., and Haas, A. R. C., *J. Biol. Chem.*, 1917, xxxii, 141.
[14] Haas, *Science*, 1917, xlvi, 462.
[15] Gustafson, F. G., *J. Gen. Physiol.*, 1918, i, 181.
[16] Brooks, M. M., *J. Gen. Physiol.*, 1918, i, 193.

COMPARATIVE STUDIES ON RESPIRATION.

V. The Effect of Ether on the Production of Carbon Dioxide by Animals.

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(Received for publication, September 6, 1918.)

The purpose of this investigation is to make a comparison of the CO_2 output of animals with that of plants under the influence of a typical anesthetic such as ether. One obstacle to such a comparative study is found in the fact that the movements of animals are more or less inhibited by anesthetics; such inhibition diminishes the CO_2 output even when it is not due to anesthetics. Hence it is difficult to say how the CO_2 output would be affected by anesthetics if this complication were absent. This difficulty is largely avoided in my studies on frog eggs and *Fundulus* embryos, while in the investigations on tadpoles and on aquatic insects it is partly obviated by the method of experimentation.

No attempt has been made to compare the results with those obtained on animals by other workers, such as those of Tashiro,[1] because the methods were so different as to render a comparison difficult.

Method.

The method used in determining the output of CO_2 is the one described by Haas.[2] The standard buffer solutions were made with mixtures of phosphates. To 10 cc. of the solution, three drops of 0.01 per cent solution of phenolsulfonephthalein were added; in all the experiments sufficient NaOH was added to bring the pH value to 8.0. As the concentration of NaOH was the same in all cases the

[1] Tashiro, S., A chemical sign of life, Chicago, 1917.

[2] Haas, A. R. C., *Science*, 1916, xliv, 105.

buffer action was identical in the solutions containing anesthetics and in the controls (the ether has practically no buffer action).

In each case 10 cc. of the solution were placed in a Pyrex glass tube of the same diameter and thickness as the Pyrex glass tubes containing the buffer solutions. The organisms were put in the solutions and the time required to change from pH 8.0 to 7.7 was recorded by means of a stop-watch. After experimentation the solutions were tested by driving out the CO_2 by means of air free from CO_2; invariably the solutions returned to pH 8.0, thus proving that no acids other than the carbonic were responsible for the change in the pH value.

The temperature was kept approximately constant at 20°C. (with variations of less than 1°C.) by means of a water bath.

In presenting the results the rate of respiration is taken as the reciprocal of the time required to change the solution from pH 8.0 to 7.7. For convenience this rate is expressed as per cent of the normal rate, which is always taken as 100 per cent. The normal rate was in most cases practically constant.[3]

Frog Tadpoles.

The first set of experiments to determine the effects of ether on the carbon dioxide output of frog tadpoles was made with ten concentrations of ether, ranging from 0.007 to 0.55 per cent by volume. The tadpoles chosen were about 20 mm. in length; at this stage, they respire through the internal gills and the skin. A single tadpole was placed in each tube containing 5 cc. of liquid, and the time required to change the solution from pH 8.0 to 7.7 was noted. Fig. 1 (Curve B) shows the effect of ether on respiration, after an exposure of 150 seconds; there was no acceleration of respiration at any concentration: on the contrary there was a decrease from 0.15 per cent ether and upward. It might be thought that this was due to the cessation of the breathing movements, by which the water is carried to the gills, or to the stopping of the muscular movements, especially of the tail. As a matter of fact, however, at these concentrations and

[3] For further details see Paper I (Osterhout, W. J. V., *J. Gen. Physiol.*, 1918, i. 171).

with this length of exposure these movements remained practically normal. The measurements of the breathing movements were made by determining the length of the time necessary to count twenty movements. In general the normal mouth movements were about twenty in 11 seconds. On comparing Curves A and B, Fig. 1, it is evident that at these concentrations the decrease in CO_2 output is

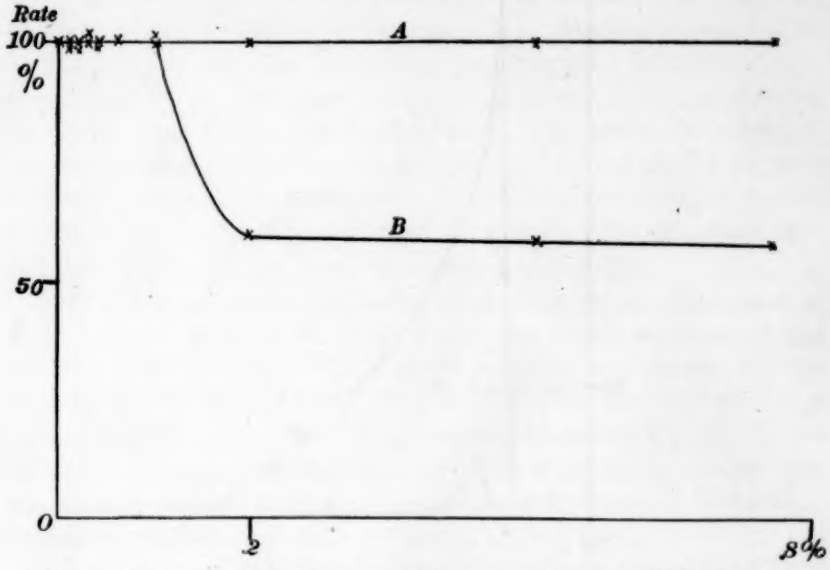

FIG. 1. Curves showing the effect of ether upon the rate of CO_2 production (Curve B) and the rate of mouth movement (Curve A) of frog tadpoles. The normal rate is taken as 100 per cent in each case. The time of exposure to ether was 150 seconds. Average of six experiments. Probable error less than 2.5 per cent of the mean. The figures expressing the per cent of ether should be corrected by multiplying by 0.73.

not due to the decrease in the water supply to the gill system. A quantitative measurement of the body movement was not possible for the reason that the movements of the tail were in many cases too rapid for accurate measurement. However, it may be stated that there was no appreciable decrease in the rate of the body movement at the concentrations in which the retardation of the CO_2 output took place. Thus, in general it may be stated that the de-

crease in CO_2 output takes place in these concentrations before the muscular movements are much affected.

Fig. 2, shows the effect of the 3.65 per cent ether (Curve A) and the effect of the 7.3 per cent ether (Curve B) on the CO_2 output. With

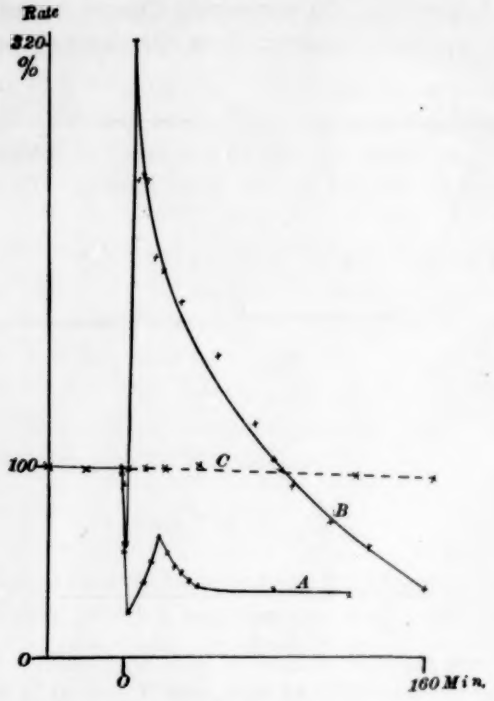

FIG. 2. Curves showing the effect of 3.65 per cent ether (Curve A), and 7.3 per cent ether (Curve B) on the CO_2 production of frog tadpoles, and control in tap water (Curve C, dotted line). The point marked 0 on the abscissa indicates the beginning of exposure to ether for Curves A and B; previous to this the material was in tap water (horizontal part of curves). The normal rate (which is taken as 100 per cent) corresponds to a change from pH 8.0 to pH 7.7 in 50 seconds for Curve A, in 43 seconds for Curve B, and in 58 seconds for Curve C. Each curve represents an average of six experiments. Probable error less than 2.5 per cent of the mean.

3.65 per cent ether a decrease of 81 per cent took place which was followed by an increase; with 7.3 per cent ether a decrease of 50 per cent occurred which was followed by an increase, the maximum of ac-

celeration being about three times the normal; in both cases decrease takes place after the increase. The reason the initial decrease is less with 7.3 per cent ether than it is with 3.65 per cent, is that the acceleration takes place more rapidly in case of the former, thus preventing the further decrease in CO_2 output.

Both 3.65 and 7.3 per cent ether caused peeling of the cuticular layers of the epidermis; this reaches a maximum in 20 minutes in 3.65 per cent ether, and in 5.5 minutes in 7.3 per cent ether. During the first 4 minutes in 3.65 per cent ether no peeling was observed. It might be supposed that this peeling causes an apparent increase in CO_2 production by allowing the CO_2 to escape more freely. However, the extent of the peeling seems to be the same in both the 3.65 and the 7.3 per cent solutions, although the acceleration in the CO_2 output in the former concentration is much greater than the latter. This difference cannot therefore be wholly due to the peeling of the skin.

Both the breathing movements and the body movements cease at these concentrations in less than 10 seconds after immersion of the tadpoles in the solutions. This might be thought to account for the drop in the rate of CO_2 output, but in view of the fact that we find a similar drop in Fig. 1, where the movement remained normal, we are obliged to conclude that the cessation of the muscular movement cannot account entirely for the falling off in the CO_2 output. This conclusion is confirmed by the experiments on frog eggs.

Aquatic Insects.

In order to avoid the disturbances due to the peeling of the epidermis, an aquatic insect was chosen. This is the common whirligig beetle, *Dineutes assimilis* Aube. It possesses a chitinous covering which prevents any peeling. 7.3 per cent ether solutions were used. Fig. 3, Curve A, shows a 94 per cent decrease in CO_2 output, after which an increase takes place followed again by a decrease.

The activity of these insects is much greater than that of tadpoles and a correspondingly greater drop in CO_2 output is to be expected as the result of anesthesia.

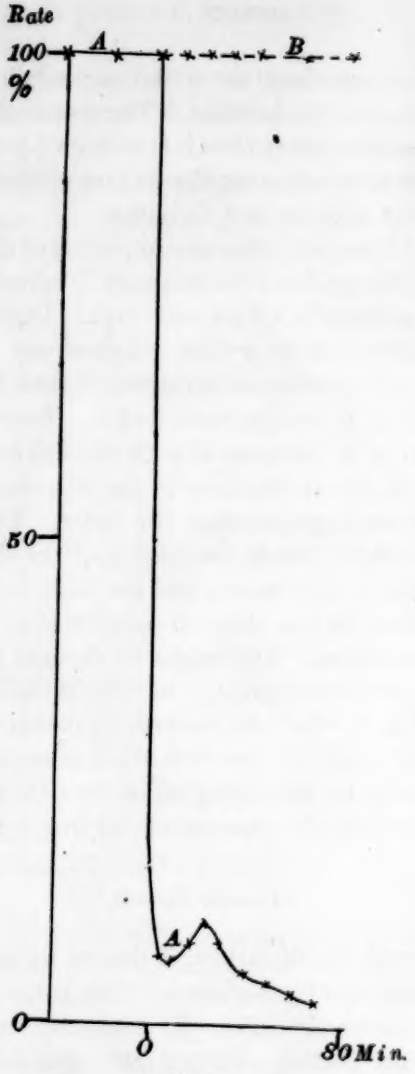

FIG. 3. Curves showing the effect of 7.3 per cent ether (Curve A) on the CO_2 production of an aquatic insect (*Dineutes*), and control in tap water (Curve B, dotted line). The normal rate is taken as 100 per cent. The point marked 0 on the abscissa indicates the beginning of exposure to ether for Curve A; previous to this, the material was in tap water (horizontal part of curves). The normal rate (which is taken as 100 per cent) corresponds to a change from pH 8.0 to pH 7.7 in 38 seconds for Curve A and in 41 seconds for Curve B. Each curve represents an average of six experiments. Probable error less than 2.5 per cent of the mean.

Frog Eggs.

In order to check the above results, frog eggs in the blastopore stage were chosen. The CO_2 output of the jelly (free from any traces of eggs) was first tested. It was found that in the time required for

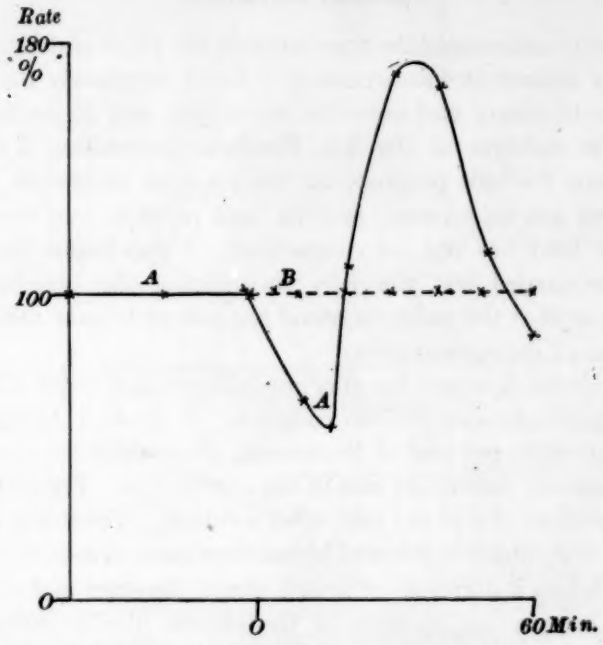

FIG. 4. Curves showing the effect of 7.3 per cent ether (Curve A) on the CO_2 production of frog eggs, and the control in tap water (Curve B, dotted line). The normal rate is taken as 100 per cent. The point marked 0 on the abscissa indicates the beginning of exposure to ether for Curve A; previous to this the material was in tap water (horizontal part of curves). The normal rate (which is taken as 100 per cent) corresponds to a change from pH 8.0 to pH 7.7 in 157 seconds for Curve A and in 142 seconds for Curve B. Each curve represents an average of six experiments. Probable error less than 2.5 per cent of the mean.

the experiments, no appreciable change in the pH value took place, thus proving that the jelly contains no organisms respiring sufficiently to interfere with the accuracy of the experiments. Fig. 4, Curve A, shows a 44 per cent retardation, followed by an increase in the rate of the CO_2 output. There was no disintegration of any portion of the

eggs which might correspond to the peeling of the epidermis in case of the tadpoles, but owing to the presence of cilia, the decrease in CO_2 output may be partly influenced by the cessation of ciliary movement as result of anesthesia.

Fundulus heteroclitus.

In order to understand the true nature of the effect of the production of CO_2 by animals it was necessary to avoid completely the disturbances due to ciliary and muscular movement, and to peeling of the skin. The embryos of the fish, *Fundulus heteroclitus*, 2 days old, were chosen for this purpose; at this stage of embryonic development there are no muscles, no cilia, and no skin, and furthermore the heart beat has not yet commenced. 1 day before the experiment was carried out, the jelly surrounding the membrane was removed, so that the embryos would not adhere to each other during the course of experimentation.

Fig. 5, Curve A, shows the effect of 0.73 per cent ether solution on the CO_2 production of the fish embryos. A gradual decrease takes place to about 46 per cent of the normal, after which the rate remains almost constant during the rest of the experiment. Fig. 5, Curve B, shows the effect of 3.65 per cent ether solution. There is a slight decrease at first, which is followed by an increase of carbon dioxide; this is succeeded by a decrease. Curve C shows the effect of 5.48 per cent ether; a tremendous increase of the carbon dioxide output takes place. If there is any decrease at the start it must be so brief as to escape observation. The rate soon reaches a maximum, after which it falls rapidly.

During the increase in 3.65 per cent and 5.48 per cent ether the embryo becomes gradually more opaque; this process begins to be observable after 20 minutes in 3.65 per cent ether and after 4 minutes in 5.48 per cent ether.

These experiments show that a decrease in the carbon dioxide output may take place as a direct effect of the ether on the protoplasmic substance, when complications due to motion are excluded. This effect may play a part in the phenomena observed in the previous experiments (on tad poles, aquatic insects, and frog eggs) where the cessation of motion is involved.

With tadpoles and *Fundulus* embryos the effect of ether at a concentration of about 0.73 per cent is reversible. The *Fundulus* embryos recover, as shown by their subsequent growth and normal development. The tadpoles also recover, as shown by their subsequent normal respiration. The increase in carbon dioxide production in 3.65

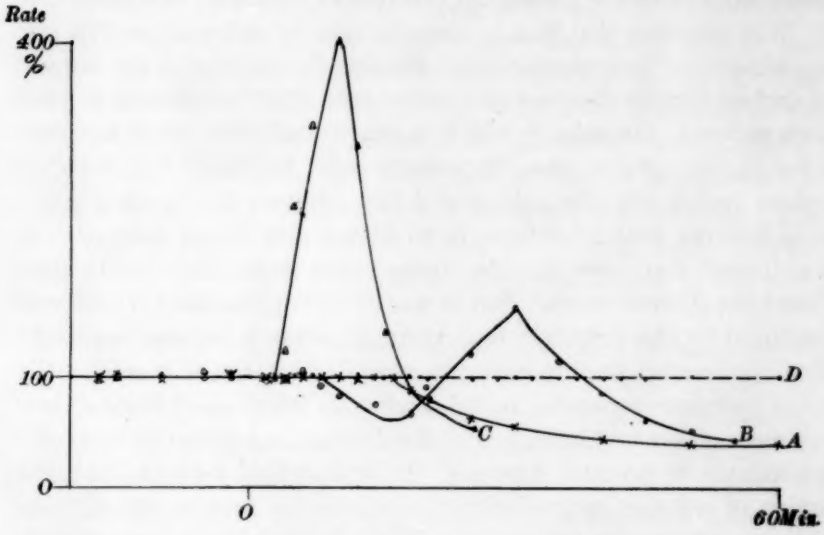

FIG. 5. Curves showing the effect of 0.73 per cent ether (Curve A), 3.65 per cent ether (Curve B), and 5.48 per cent ether (Curve C), on the CO_2 production of *Fundulus* embryos, and control in tap water (Curve D, dotted line). The point marked 0 on the abscissa indicates the beginning of exposure to ether for Curves A, B, and C; previous to this, the material was in tap water (horizontal part of curves). The normal rate (which is taken as 100 per cent) corresponds to a change from pH 8.0 to pH 7.7 in 128 seconds for Curve A, in 119 seconds for Curve B, in 176 seconds for Curve C, and in 111 seconds for Curve D. Each curve represents an average of six experiments. Probable error less than 2 per cent of the mean.

and 7.3 per cent ether is accompanied by irreversible changes leading to death. If *Fundulus* embryos were removed from the ether about 75 seconds after the increase began in 3.65 per cent ether, they failed to recover. In tadpoles the recovery is no longer possible 47 seconds after the increase has begun. In frog eggs similar results were obtained.

It is evident from these experiments that in animals the effect of ether is of two types: (1) the decrease in the output of carbon dioxide, which is reversible; (2) the increase of the output of CO_2 which is irreversible.

It was shown by Loeb and Wasteneys[4] that 1 per cent ether produces narcosis in the 1 week old embryos of *Fundulus*, and there can be little question that this is also the case in embryos used in my experiments. The question arises whether the decrease in the output of carbon dioxide observed in 0.73 per cent ether is sufficient to produce narcosis. In order to test this, experiments were made to determine to what degree the temperature must be lowered in order to reduce carbon dioxide output of 2 day embryos to the same point as in 0.73 per cent ether (that is, to 57 per cent of the normal). It was found that lowering the temperature from 22 to 13°C. produced the desired result. But it was observed that no narcosis was produced by this drop in temperature, for when 8 day embryos were similarly treated, their movements remained practically normal. We must therefore conclude, as did Loeb and Wasteneys[5] from an experiment on sea urchin eggs, that the decrease in respiration is wholly inadequate to produce narcosis. It is therefore evident that the action of anesthetics producing narcosis is due to some other cause than the effect upon respiration. This is in complete agreement with the striking experiments of Loeb and Wasteneys[4] on *Fundulus* embryos, where they found that oxidation must be reduced to one-fourteenth of the normal in order to produce narcosis.

A comparison of the data obtained by the writer on the effect of ether on the carbon dioxide production of tadpoles, aquatic insects, frog eggs, and killifish eggs with those described in the preceding articles of this series, as well as with those obtained by Haas,[6] shows that there exists a difference in plants and animals as regards the effect of ether. With animals, in weaker concentrations, such as 0.73 per cent solution of ether, a decrease in the CO_2 output takes place, and the effect is reversible, while in stronger concentrations, such as 3.65

[4] Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1913, lvi, 295.
[5] Loeb and Wasteneys, *J. Biol. Chem.*, 1913, xiv, 517.
[6] Haas, *Science*, 1917, xlvi, 462.

per cent and 7.3 per cent ether, an increase in the carbon dioxide production takes place, the effect being irreversible. This is different from the effects usually found in plants.

SUMMARY.

1. The experiments on frog tadpoles show that with 0.15, 0.37, and 0.55 per cent ether solutions there is a decrease in CO_2 output. The effect is reversible. With these concentrations the breathing movements and body movements remained normal during the experiment. In 3.65 and 7.3 per cent ether there is a decrease of respiration followed by an increase which in turn is followed by a decrease. The increase may reach about three times the normal rate. The increase in the CO_2 output is accompanied by the peeling of the skin. The effect is irreversible.

2. Experiments on an aquatic insect, *Dineutes assimilis* Aube, show that in 7.3 per cent ether there is a decrease followed by an increase which in turn is followed by a decrease. There is no apparent disintegration of structures in the organism accompanying the increase. The effect is irreversible.

3. The experiments on frog eggs with 7.3 per cent ether show a result similar to that found in aquatic insects.

4. Experiments on *Fundulus* embryos show that with 0.73 per cent ether there is a reversible decrease in the rate of CO_2 production. In 3.65 per cent ether there is a temporary decrease followed by an increase, after which the rate begins to fall off. In 7.3 per cent ether there is an immediate increase amounting to 307 per cent which is followed by a decrease. The increase in the 3.65 and 7.3 per cent ether is accompanied by irreversible changes leading to death. The decrease found in 0.73 per cent ether is not sufficient to cause narcosis, as is shown by experiments on which the same decrease is produced by lowering the temperature.

5. These experiments show that narcosis is not due to asphyxia. The action of anesthetics is due to some other cause than the effect on respiration.

There is a difference between the animals studied and the plants described in this series of articles, since in animals the increase in the

CO_2 output is accompanied by irreversible changes leading to death, while this is not necessarily the case in plants. The reversible (narcotic) action of ether on the animals studied was accompanied by a decrease in the carbon dioxide output; in plants this is not ordinarily the case.

These facts are of considerable interest, but their interpretation must be left to future investigation.

ON THE CONTROL OF ROPE IN BREAD.*

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(Received for publication, August 27, 1918.)

Rope is a condition of bacterial decomposition of bread which leads to a peculiar slimy or ropy consistency of portions of the interior of the loaf, produces an odor resembling that of ensilage, and renders the loaf unacceptable. During summer months the loss of bread through the development of rope may be very great, and individual bakeries may become so thoroughly infected that they can be operated only with great difficulty.

Several closely related organisms have been isolated from ropy bread, some or all of which are undoubtedly the effective agents of the decomposition.[1–3]

All varieties of bread appear to be liable to this decomposition within 24 hours or more after baking, but it has been stated that those containing a considerable quantity of material other than white flour are most often infected. For many years it has been customary to add lactic acid, acetic acid, or some other acid substance to the dough as a means of repressing the growth of the organism. Under favorable circumstances this method of treatment may be very satisfactory; but it is always carried out by rule of thumb, and there is at present

* Published with the permission of the Surgeon General.

[1] Lafar, F., Handbuch der technischen Mykologie, Jena, 1905–08, ii, 520.

[2] Fuhrmann, F., Vorlesungen über technische Mykologie, Jena, 1913, 257; *Centr. Bakteriol., 2te Abt.*, 1906, xv, 385, 538.

[3] Beattie, J M., and Lewis, F. C., *Lancet*, 1917, ii, 211

no general agreement as to the kind or quantity of acid that should be employed, or the scientific explanation of its action.

The present investigation has been upon a strain of *Bacillus mesentericus* isolated from a ropy loaf and characterized by one of us. In addition three other cultures, from two other sources, have been examined and appear to be identical.

The bacteriological study of the bacillus, which belongs to the *Bacillus mesentericus* group, is not completed and there has been no opportunity for comparison with cultures from other sources. Since a number of bacteria, all probably closely related, have been described as causes of ropy bread and since the bacteriological data are meager and not recent, it seems best, for the present, not to name the organism isolated in this vicinity. Its relationship to the production of characteristic rope has been proved beyond question, and the four cultures from three different local sources are identical.

The bacillus is a motile, Gram-positive, spore-bearing bacillus. In 24 hour cultures on plain agar its dimensions average 2.5 microns by 0.5 to 0.75 microns. The ends of the rods are rounded. The spores are ovoid in shape and are situated approximately in the middle of the bacilli. The width of the spores slightly exceeds that of the rods and they measure 0.5 to 1.75 microns by 0.75 to 0.85 microns. The spores are produced in great abundance in cultures less than 24 hours old and are extremely resistant to boiling.

The bacillus is a strict aerobe. It grows readily on all ordinary media, resulting in a decrease of acidity. Coagulated blood serum is completely liquefied. Litmus milk is rapidly curdled and liquefied. The litmus is reduced in the depths of the medium but at the surface the color becomes deeper blue. Cultures in agar are odorless, but in wheat flour media the characteristic odor of ropy bread is produced.

The colonies on agar media are flat, finely granular, dull white or grayish, with irregular margins. In 24 hours the colonies become wrinkled or thrown into coarsely irregular elevations, and the margins become convoluted or arborescent. Colonies several days old develop a brownish tint which is more marked upon media containing carbohydrates. On liquid media a wrinkled and convoluted pellicle is formed; turbidity develops slowly, and begins beneath the surface pellicle.

The optimum temperature is about 38°C. At ordinary room temperatures (20–24°C.) growth is very slight.

The action on bread was studied by the inoculation of baked loaves and by the incorporation of cultures in the dough. In both cases the bread was incubated at 37.5°C. and examined after 24 or 36 hours. The rope produced by addition of the pure cultures was identical in physical properties and odor with that of the bread from which the cultures were isolated.

In all instances the softening occurred just in the centers of the loaves and progressed outwards. The foci of softening in bread took on a brownish tint and became sticky, and particles on separation yielded delicate threads of from a few inches to 6 or 7 inches in length. In the case of cultures introduced with considerable amounts of liquid media a cavity was produced. In bread or dough to which acid was added the softening and odor were less pronounced and were finally suppressed at a hydrogen ion concentration of approximately 10^{-5}N. In acidities sufficient to retard the growth of the organism the discoloration of the softened foci did not occur.

The original loaf was softened and discolored, and slowly developed the characteristic slimy and viscous condition. Examination of an aqueous extract of a decomposed portion of this loaf with the concentration cell revealed a marked diminution of acidity,—from approximately pH 5.3, characteristic of normal bread, to pH 6.2. Other specimens gave the following values for pH; 6.5, 6.1, 6.3.

As a result of these observations we were led to a study of the relation between hydrogen ion concentration and the growth of the organism.

Three culture media were prepared: a synthetic medium known as Cohn's solution, a potato medium, and a wheat paste. These media were then divided into portions, acid or alkali was added to bring these to different hydrogen ion concentrations, they were sterilized, and finally inoculated with a culture of the organism, or of the spores.

The accompanying curves of the variation in hydrogen ion concentration of these media during titration will indicate the general characteristics of the preparations, and may perhaps serve others as a basis for similar work.

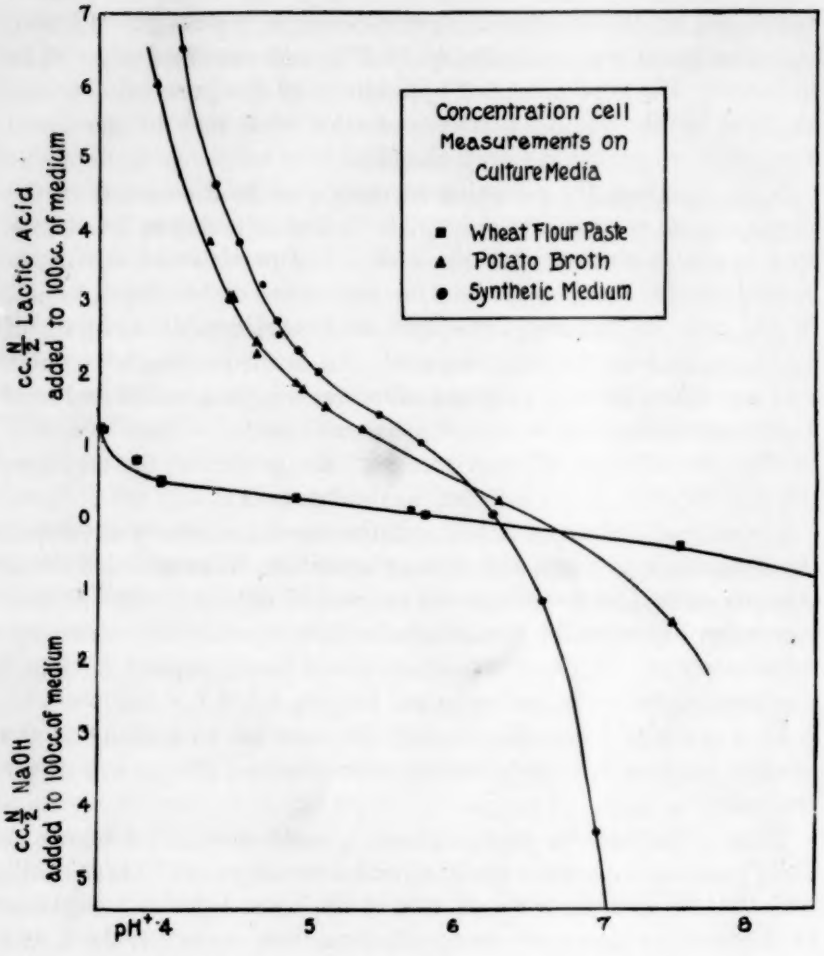

Concentration cell
Measurements on
Culture Media
Wheat Flour Paste
Potato Broth
Synthetic Medium
cc. N/2 Lactic Acid
added to 100cc. of medium
cc. N/2 NaOH
added to 100cc. of medium
pH
4
5
6
7
8

FIG. 1.

Preparation of the Media.

Cohn's Medium with the Addition of Asparagine.

K_2HPO_4	5.0 gm.
$Ca_3(PO_4)_2$	0.5 "
$MgSO_4$	5.0 "
Ammonium tartrate	10.0 "
Asparagine	4.0 "
Distilled water	1,000.0 cc.

The ingredients were weighed and dissolved in the water and after tubing were sterilized in the autoclave at 15 pounds pressure for 30 minutes.

Wheat Bouillon.

Wheat flour	15.0 gm.
$MgSO_4$	0.5 "
KNO_3	1.0 "
Dextrose	15.0 "
Distilled water	1,000.0 cc.

The wheat flour was added after solution of the other ingredients and the whole boiled for a few minutes, tubed, and sterilized in the autoclave at 15 pounds pressure for 30 minutes.

Potato Broth.—Two parts of water to one of freshly grated raw potatoes were mixed and allowed to stand for 24 hours. The mixture was then strained through cloth and the liquid filtered through coarse filter paper, tubed, and sterilized at 15 pounds pressure for 30 minutes. It was not considered of importance to avoid the precipitate which occurred.

The cultures were incubated for 5 days; meanwhile they were observed daily, and finally both inoculated tubes and controls were examined for their hydrogen ion concentrations. The results of this experiment are presented in Table I. It should be noted that the results with the wheat paste were uncertain. This appears to be due to its lack of homogeneity, and low buffer content. It is also to be observed that the spores are, as might be expected, somewhat more restricted in growth than the organisms themselves.

A second experiment was performed over a narrower range of hydrogen ion concentration, the results of which, presented in Table II, agree satisfactorily with those of the first experiment.

From these experiments it seems safe to conclude that a hydrogen ion concentration sensibly higher than 10^{-5}N completely inhibits the growth of *Bacillus mesentericus*.

TABLE I.

Growth of Rope at Different Hydrogen Ion Concentrations.

Experiments 668 and 685.

Tube No.	Solution added to 100 cc. of medium.	Organisms: Day.					Spores: Day.					pH after incubation.		
		1st	2nd	3rd	4th	5th	1st	2nd	3rd	4th	5th	Control.	Organisms.	Spores.
	Wheat flour paste.													
	cc.													
1	6.0 0.1 N $C_3H_4O_3$	0	0	0	0	0						3.53		
2	4.0 0.1 " "	0	0	0	0	0						3.77	3.63	
3	2.4 0.1 " "	0	0	0	0	0						(5.22)	3.94	
4	1.3 0.1 " "	0	+	+	+	+	0	?	+	+	+	4.87	4.40	5.45
5	0.4 0.1 " "	0	+	+	+	+	0	+	+	+	+	5.68	6.62	5.47
6	0.3 0.1 " NaOH	0	+	+	+	+	0	+	+	+	+	5.82	5.58	5.80
7	0.8 0.1 " "	0	++	++	++	++	0	+	+	+	+	6.05	5.41	5.73
8	1.4 0.1 " "	0	++	+++	+++	+++	0	+	++	++	++	5.99	5.16	5.54
9	2.7 0.1 " "	0	+	+++	+++	+++	0	+	++	++	++	5.92	5.06	5.83
10	4.4 0.1 " "	0	+	+	+	+	0	+	+	++	++	5.60	5.49	5.88
	Cohn's solution.													
12	12.8 0.5 N $C_3H_4O_3$	0	0	0	0	0						3.82	3.75	
13	6.5 0.5 " "	0	0	0	0	0						4.07	4.12	
14	2.5 0.5 " "	0	0	0	0	0	0	0	0	0	0	4.75	4.72	4.77
15	1.0 0.5 " "	0	++	++	++	++	0	0	0	0	0	5.74	6.92	5.46
16	0	0	+	++	++	++	0	0	0	0	0	6.25	7.08	6.23
17	1.2 0.5 " NaOH	0	+	+	+	++	0	0	0	0	0	6.60	7.74	6.39
18	4.4 0.5 " "	0	+	+	+	+	0	0	++	++	++	6.98	7.94	7.93
19	9.1 0.5 " "	0	0	0	+	+	0	+	+	+	++	7.14	8.11	8.17
20	11.2 0.5 " "	0	0	0	0	+	0	+	+	+	+	7.64	8.21	8.20

TABLE I—*Concluded.*

								Potato broth.							
22	12.0	0.5 N	$C_2H_6O_2$	0	0	0	0	0						3.39	3.53	
23	6.0	0.5 "	"	0	0	0	0	0						3.90	3.94	
24	4.0	0.5 "	"	0	0	0	0	0	0	0	0	0	0	4.09	4.08	4.19
25	3.0	0.5 "	"	0	0	0	0	0	0	0	0	0	0	(4.52)	4.40	4.39
26	2.25	0.5 "	"	0	+	+	+	+	0	0	0	0	0	4.59	7.01	4.68
27	1.75	0.5 "	"	0	+	+	+	+	0	+	+	+	+	4.92	6.63	7.37
28	1.20	0.5 "	"	+	+	++	++	++	+	+	++	++	++	4.93	7.10	7.30
29	0.20	0.5 "	"	+	++	+++	+++	+++	+	+	++	++	++	6.28	7.05	7.60
30	1.50	0.5 "	NaOH	++	+++	+++	+++	+++	+	+	+	+	++	7.49	7.57	7.65

In order to test this conclusion upon bread itself a series of loaves of different hydrogen ion concentrations were prepared with the co-operation of Messrs. C. F. Hathaway and Sons of Cambridge. These

TABLE II.

Growth of Rope at Different Hydrogen Ion Concentrations.
Experiments 686 and 700.

Tube No.	Solution added to 100 cc. of medium.			Organisms: Day.					Spores: Day.					pH after incubation.		
				1st	2nd	3rd	4th	5th	1st	2nd	3rd	4th	5th	Control.	Organisms.	Spores.
				Wheat flour paste.												
	cc.															
1	1.4	0.1 N	$C_3H_6O_3$	+	+	++	++	++	0	+	+	+	+	5.12	5.78	5.57
2	1.15	0.1 "	"	+	+	++	++	++	0	+	+	+	+	5.22	5.55	6.78
3	0.90	0.1 "	"	+	+	++	++	++	0	++	++	++	++	5.66	5.75	5.78
4	0.70	0.1 "	"	+	++	++	++	++	0	+	+	+	+	5.64	5.97	5.97
5	0.55	0.1 "	"	+	++	++	++	++	0	+	+	+	+	6.40	5.68	6.63
6	0.45	0.1 "	"	+	++	++	++	++	0	+	+	+	+	6.30	5.71	6.43
				Cohn's solution.												
12	4.6	0.5 N	$C_3H_6O_3$	0	0	0	0	0	0	0	0	0	0	4.30	4.33	4.34
13	3.8	0.5 "	"	0	0	0	0	0	0	0	0	0	0	4.46	4.46	4.46
14	3.2	0.5 "	"	+	+	+	+	+	0	0	0	0	0	4.63	4.61	4.61
15	2.7	0.5 "	"	+	++	++	++	++	0	0	0	0	0	4.73	6.84	4.73
16	2.3	0.5 "	"	0	0	0	0	0	0	0	0	0	0	4.89	4.91	4.90
17	2.0	0.5 "	"	+	++	++	++	++	0	0	0	0	0	5.03	7.19	5.03
18	1.4	0.5 "	"	+	+	+	+	+	0	0	0	0	0	5.45	7.39	5.50
				Potato broth.												
21	3.8	0.5 N	$C_3H_6O_3$	0	0	0	0	0	0	0	0	0	0	4.27	4.25	4.25
22	3.0	0.5 "	"	0	0	0	0	0	0	0	0	0	0	4.42	4.43	4.41
23	2.4	0.5 "	"	0	0	0	0	0	0	0	0	0	0	4.59	4.64	4.61
24	1.85	0.5 "	"	0	+	++	++	++	0	0	++	++	++	4.85	6.59	6.51
25	1.50	0.5 "	"	0	+	++	++	++	+	+	+	+	+	5.07	6.33	7.25
26	1.20	0.5 "	"	+	++	++	++	++	+	+	+	+	+	5.28	7.52	7.24

loaves were then inoculated with a culture of the organism and incubated in a moist condition at a temperature of 37.5°C. The results of this experiment are presented in Table III.

TABLE III.

Growth of Rope in Bread. Inoculation After Baking.

Loaf No.	pH	Growth of organisms.
12	4.31	0
9	4.55	0
5	4.77	0
8	4.81	+
10	4.87	0
11	5.07	+
6	5.09	+
7	5.25	+
1	5.28	0*
2	5.35	++
3	5.44	++
4	5.47	++

* Large buffer content.

In order to reproduce more closely the actual conditions of baking another series of loaves were baked in our own laboratory from dough to which 1 cc. of a culture of the organism per loaf of bread had been added. The results of this experiment are given in Table IV.

TABLE IV.

Growth of Rope in Bread. Inoculated Before Baking.

Loaf No.	0.5 N lactic acid added to dough.	pH		Growth.
		Before baking.	After baking.	
	cc.			
1	0	5.30	5.38	++
2	1.0	5.24		+
3	1.6			+
4	2.4			?
5	3.2	4.98		0
6	4.0	4.94	4.98	0

Taking account of these facts and of the observation which we have made in another connection, that wheat substitutes are commonly less acid than wheat flour and often contain larger amounts of buffer substances, it seems safe to conclude, first, that the development of

rope depends upon the existence of a hydrogen ion concentration in bread sensibly less than about 10^{-5}N, secondly, that such a condition is likely to exist in war bread or other loaves which contain something besides white wheat flour, and thirdly, that the action of acid as a preventive of rope depends primarily upon the increase of the hydrogen ion concentration.

These investigations clearly show that the growth of *Bacillus mesentericus* is a function of the hydrogen ion concentration of the medium and that it is favored by the diminished hydrogen ion concentration which is due to the products of its own metabolism. This change in the hydrogen ion concentration may serve as a measure of the growth of the organism and as a means of identification.

It is also interesting to observe that the growth of yeast tends to produce a very acid reaction,—perhaps pH 4.5. Such a reaction effectively checks the development of *Bacillus mesentericus* as well as of most other organisms. Unquestionably this peculiarity of the metabolism of yeast has much to do with the ease with which pure cultures of yeast are obtained, preserved, and employed in baking and brewing. For example, the 12 hour sponge process of bread making may sometimes effectively safeguard the bread. It must not be supposed, however, that an acid culture of yeast is necessarily free from spores.

As a result of this investigation it seems clear that, when possible, the hydrogen ion concentration of bread should be maintained near 10^{-5}N. Our observations upon ropy bread, both in the laboratory and in the bakery, seem to indicate that such practice would prevent the occurrence of rope. The reaction of dough and bread may be controlled with the help of a suitable indicator, and perhaps more conveniently by the use of a buffer mixture consisting of lactic acid and a lactate or acetic acid and an acetate.

At present the simplest method of controlling the reaction of dough or bread consists in the use of methyl red. When this indicator is colored red rather than orange, we believe the reaction to be sufficiently acid to prevent the development of rope.

THE RATE OF TRANSMISSION IN THE NERVE NET OF THE CŒLENTERATES.

By G. H. PARKER.

(*Contributions from the Zoölogical Laboratory of the Museum of Comparative Zoölogy, Harvard College, Cambridge, No. 313.*)

(Received for publication, September 2, 1918.)

Direct measurements of the rate of transmission in the nerve net of the cœlenterates seem never to have been undertaken heretofore. Romanes (1878) and Harvey (1912) have both recorded the rate of the contraction wave in medusæ and, while it is probable that this wave, as it can be seen coursing around the jellyfish bell, represents the rate of nervous transmission, this method of determining such a rate is at best indirect. In sea anemones the nervous and muscular elements for some of their more striking responses are so much more separated anatomically than they are in the jellyfishes that an independent determination of the rate of nervous transmission is possible. This is particularly true of *Metridium marginatum*, whose large size permits the preparation of considerable lengths of tissue through which transmission may be accomplished.

It is well known that when the pedal edge of the column of *Metridium* is stimulated, the animal quickly responds by a vigorous contraction of the longitudinal mesenteric muscles, by which the oral disc is retracted. This response occurs without the appearance of muscular activity in the region intervening between the point of stimulation and the responding muscles, and hence this region must be assumed to be bridged over through the activity of nervous tissue. Moreover, the pedal edge of the column of *Metridium* may be partly cut off as a long tongue of tissue, one end of which can be left still attached to the animal (Fig. 1). When the free end of this tongue, which may be 10 to 15 cm. in length, is stimulated, the animal responds in the characteristic way but without exhibiting muscular contraction on the length of the tongue itself. This portion of the

animal contains a nerve net and hence may serve as a region in which to measure the rate of transmission in that type of nervous tissue.

The method employed in this procedure was that long since used in the measurement of nerve rates. A stimulus was applied to the distal end of the tongue (Fig. 1, *2*), and the time intervening between the moment of application and the moment of response was measured. The stimulus was next applied at the root of the tongue (Fig. 1, *3*), and the time between the moment of application and the moment of response was again measured. The difference between the amounts of time consumed in these two operations represents the time necessary for the transmission of an impulse from the distal end of the tongue

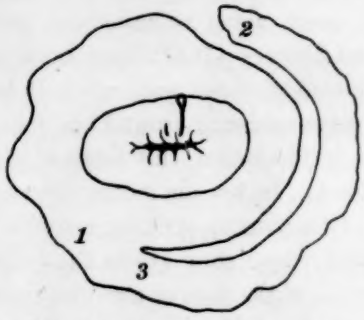


FIG. 1. Oral view of a *Metridium* showing the tongue of tissue cut from the pedal margin of the column, the three points of stimulation, *1*, *2*, and *3*, and the region of the lip into which the hook was inserted.

to its root, a distance that is easily measured and that thus affords a basis for the determination of a rate.

The records of the several operations necessary in these measurements were made in the usual way on a kymograph. Time was recorded by a marker actuated by an electrically driven tuning fork at the rate of 50 vibrations per second. The moment of applying the stimulus was recorded by a second marker which was activated electrically from the same switch through which the stimulating current was sent. This consisted of a faradic current that was disagreeably strong to the human finger. It was applied by appropriate electrodes to the portion of the pedal edge of the sea anemone that was to be stimulated. This edge, as is well known, is extremely

sensitive to mechanical contact but only moderately so to electrical stimulation. It was, therefore, important to place the electrodes in position without disturbing the animal and to stimulate with the electric current only after all possibility of mechanical disturbance had been passed. The response of the animal was recorded on the drum of the kymograph by a delicate heart lever which was attached by a thread and hook to the lip of the sea anemone. As a matter of good fortune the lip of *Metridium* is extremely insensitive and a hook may be passed through it and drawn up lightly without calling

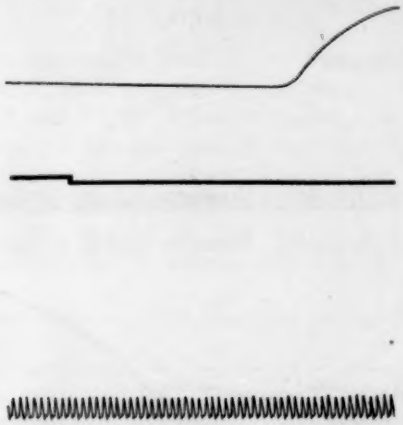


FIG. 2. Kymograph record in which the lowest line represents time in fiftieths of a second, the middle line the moment of application of the stimulus, and the uppermost line the response of the sea anemone. The stimulus was applied at position *1* (Fig. 1).

forth any particular response on the part of the animal. With this attachment the slightest contraction of the longitudinal muscles of the mesenteries was easily observed.

Large specimens of *Metridium* were allowed to attach themselves to sheets of glass about 15 cm. square. After they had firmly fixed themselves, each one was subjected to the following procedure. After the necessary connections between the animal and the kymograph were made, an electric stimulus was applied to the pedal edge of the column and the normal reaction time, as indicated by the retraction of the oral disc, was determined (Fig. 2). The long tongue

of tissue was then cut from the pedal edge (Fig. 1). After the sea anemone had reexpanded, the stimulus was applied to the distal end of this tongue and the time between the moment of application of

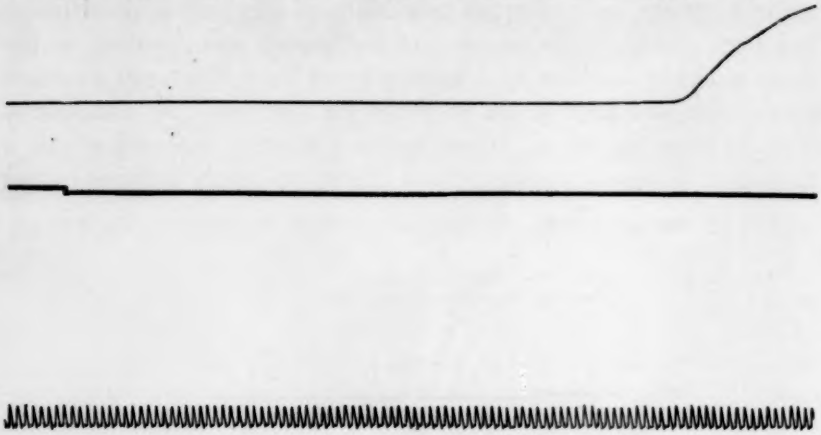
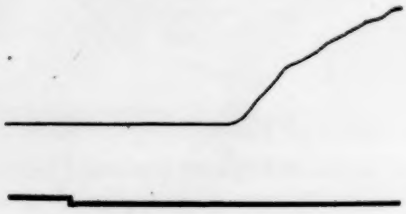


FIG. 3. Kymograph record. Stimulus applied at position *2* (Fig. 1).



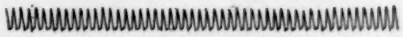


FIG. 4. Kymograph record. Stimulus applied at position *3* (Fig. 1). This record is from a set not included in Table I.

the stimulus and the moment of response was determined (Fig. 3). The stimulus was next applied to the base of the tongue and a second determination was made (Fig. 4). Three sets of these three deter-

minations were made from each animal. After these steps had been taken the glass plate to which the sea anemone was attached was turned over and the length of the tongue from one point of stimulation to the other was measured. In preparing the tongue it was found easy to leave the tissue attached to the glass plate to which the sea anemone as a whole had attached itself and thus to facilitate greatly the measurement of the length of the tongue. The results of these measurements on three animals, A, B, and C, are given in Table I.

TABLE I.

Reaction times are expressed in hundredths of a second of three specimens of *Metridium*, A, B, and C, to faradic stimuli applied to the pedal edge of the column at points *1*, *2*, and *3* (Fig. 1) and responded to by the contraction of the longitudinal mesenteric muscles. The readings were made in fiftieths of a second, but for convenience in tabulation they have been converted into hundredths of a second. The rates of transmission, as calculated from data contained in the table, are given for the three animals in the lowest line in the table. During experimentation the animals were kept in sea water at a temperature of 21°C.

Specimen.	A		B		C	
	Observations.	Averages.	Observations.	Averages.	Observations.	Averages.
	sec.	*sec.*	*sec.*	*sec.*	*sec.*	*sec.*
Reaction time from point *1*.	0.60		0.64		0.56	
	0.62		0.62		0.52	
	0.58	0.600	0.66	0.640	0.58	0.553
Reaction time from point *2*.	1.48		1.98		1.40	
	1.36		1.66		1.50	
	1.64	1.493	2.04	1.893	1.46	1.453
Reaction time from point *3*.	0.56		0.66		0.54	
	0.68		0.58		0.60	
	0.60	0.613	0.68	0.640	0.50	0.547
Time between points *2* and *3*, *sec.*		0.880		1.253		0.906
Distance from point *2* to point *3*, *mm.*		119		152		132
Rate per sec., *mm.*		135+		121+		146−

In Specimen A the average reaction time over the tongue of tissue was found to be 1.493 seconds and from the root of the tongue 0.613 seconds, which agrees very closely with that of the intact animal, 0.60 seconds. The time consumed in transmission over the tongue is the difference between 1.493 and 0.613 or 0.880 seconds. As the length of the tongue between the points stimulated was 119 mm., it follows that the rate of transmission was a little over 135 mm. per second. By a similar method of procedure the rate in Specimen B was determined to be 121 mm. per second, and in Specimen C 146 mm. per second.

These rates are relatively low compared with even the lower rates already determined for transmission in the nerve fibers of metazoans, but this is not surprising, for the nerve net has generally been supposed to be a less rapid and efficient organ of transmission than the nerve trunk. The rate is lower than that given by Romanes (1878) for what he calls the stimulus wave in *Aurelia*, namely 9 inches or about 229 mm. per second, and is much lower than that given by Harvey (1912) for the contraction wave in *Cassiopea*, some 775 mm. per second. In both these instances, however, the rates measured refer to waves of muscular activity and though these waves very probably reflect accurately the rate of nervous transmission, they are not free from complications with possible muscular transmission, whereas the rates derived from *Metridium* are. The measurements taken on *Cassiopea* were made at Dry Tortugas, Florida, where the sea water is relatively warm, a condition which, as is well known, increases the rate of transmission. At Woods Hole, Massachusetts, where the measurements on *Metridium* were made, the sea water in which the sea anemones were immersed during experimentation had a temperature of 21°C.

CONCLUSION.

Nerve net transmission in *Metridium* at 21°C. varies from 121 to 146 mm. per second.

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AMPHOTERIC COLLOIDS.

II. Volumetric Analysis of Ion-Protein Compounds; the Significance of the Isoelectric Point for the Purification of Amphoteric Colloids.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, August 28, 1918.)

INTRODUCTION.

In order to understand the influence of the hydrogen ion concentration upon the chemical and physical behavior of proteins we may well keep in mind the rôle of the hydrogen ion concentration upon the reactions of an amphoteric metal hydroxide, like aluminium or chromic hydroxide, whose chemical constitution is better known than that of the proteins.

"Aluminium hydroxide dissolves in acids. From its solution in hydrochloric acid, an aluminium salt, aluminium chloride $AlCl_3$, $6H_2O$, is obtained. It also combines with strong bases, dissolving for instance in a solution of sodium hydroxide and forming an aluminate, $NaAlO_2$.... According to the best knowledge we have on the subject, the molecule of aluminium hydroxide has the following structure or arrangement of its atoms $Al(-O-H)_3$. It is readily seen that the cleavage of the molecules may produce, either aluminium and hydroxide ions, characteristic ions of a base, or aluminate and hydrogen ions, characteristic ions of an acid:

$$Al^{+++} + 3\,\overline{O}H \rightleftarrows Al(-O-H)_3 \rightleftarrows Al\overline{O}_2 + H^+ + H_2O."[1]$$

It is obvious that between the action of acids and of bases producing these two types of electrolytic dissociation there must be one hydrogen ion concentration in which aluminium hydroxide is practically neither able to form Al^{+++} nor $Al\overline{O}_2$ ions, and this would be the isoelectric point.

[1] Stieglitz, J., The elements of qualitative chemical analysis, New York, 1911, 171.

We may apply the experience gained from aluminium hydroxide to proteins which are also amphoteric electrolytes and for some of which the exact location of the isoelectric point is known. For gelatin it lies at pH = 4.7. In a previous paper[2] it was shown that when pH > 4.7, gelatin is only able to dissociate as gelatin (capable of combining with metals to form metal gelatinates, *e.g.* sodium gelatinate); when pH < 4.7, gelatin is only capable of dissociating as gelatin (capable of combining with anions to form, *e.g.*, gelatin chloride). At the isoelectric point it is practically not dissociated at all. There is this difference between amphoteric electrolytes of the type of proteins and that of metal hydroxides, namely that in the case of proteins the OH ions and the H ions are attached to different groups in the molecule.

We also have shown that a metal gelatinate must give off its metal ion when the hydrogen ion concentration is raised beyond that of the isoelectric point; and that a gelatin-anion compound must give off its anion when the hydrogen ion concentration is lowered below that of the isoelectric point. At the isoelectric point gelatin cannot be in combination with either anion or cation and such gelatin free from *ionogenic* impurities we designate as pure gelatin. It would thus appear that we can free proteins and amphoteric colloids in general from *ionogenic* impurities by bringing the ampholyte to its isoelectric point. Such impurities, however, as form part of the complex gelatin ion can, of course, not be removed by this method. In this and some following papers we intend to furnish additional proof for the correctness of these ideas. This can be done by making the gelatin combine with such ions as Ag or Br or CNS, etc., whose quantity can be easily determined volumetrically.

The Combination of Cation with Gelatin.

In all experiments in which the chemical or physical influence of an electrolyte upon an amphoteric colloid is to be investigated it is necessary to remove the excess of electrolyte after it has had time to act on the colloid; and the writer has shown that the neglect of this precaution has caused a good deal of the confusion which prevails in the

[2] Loeb, J., *J. Gen. Physiol.*, 1918, i, 39.

literature of colloid chemistry. By using gelatin in the condition of a fine powder we are able to wash away the excess of electrolyte after it has had time to act on the gelatin.

When we put 1 gm. of powdered gelatin for 30 minutes at 20 or 15° into 100 cc. of 3 M/1024 HCl or HNO_3, and then wash the gelatin on the filter to remove the excess of acid, we obtain a gelatin with pH of about 4.7; *i.e.*, of the isoelectric point. When we use a higher concentration than 3 M/1024 HCl, we get a pH < 4.7, and a gelatin which is on the acid side of the isoelectric point and which is able to act only as cation; when we use an acid less concentrated than 3 M/1024, we get after the excess of acid is washed away a gelatin whose pH > 4.7 and which being on the alkaline side of its isoelectric point can act only as an anion and can combine only with a cation.

In order to get clear results it is well to work with a series of different concentrations of an acid, *e.g.* M/4, M/8, M/16, etc., to M/8192 HCl or HNO_3, so that we obtain series of gelatin solutions with different pH on either side of the isoelectric point. After the excess of acid is washed away by several perfusions with H_2O, the gelatin is perfused three times with 25 cc. of the same solution of a salt, *e.g.* M/8 NaBr or M/8 NaCNS or M/16 $AgNO_3$, to bring about a reaction between salt and gelatin. It is necessary to stir up the gelatin on the filter while the salt solution is poured on to bring about as complete a contact between the powdered particles of gelatin and salt as possible. After the salt solution has drained off, the excess of salt is washed away by six perfusions with 25 cc. of H_2O each. The gelatin is then melted and made into a 1 per cent solution whose pH is determined and which is then analyzed for Br or silver, as the case may be. We shall describe some of the results obtained by this method.

1 gm. of finely powdered gelatin is put for 30 minutes at 15°C. into each of a series of beakers containing 100 cc. of HNO_3 varying from M/8 to M/8192; and into one beaker containing 100 cc. of distilled water serving as a control. The gelatin is then put on a filter, washed, and is perfused three times with 25 cc. of M/16 $AgNO_3$ in the way described, and subsequently six times with 25 cc. of H_2O to wash away the excess of $AgNO_3$. The swelling is measured while the gelatin is still in the cylindrical funnel, and the gelatin is melted and made into a 1 per cent solution. This part of the experiment is of course carried

out in a dark room. Then the pH is determined, and 25 cc. of the solution are used to determine the quantity of silver in combination with the gelatin, according to Volhard's method.

Fig. 1 contains two curves the abscissæ of which are the logarithms of the concentrations of HNO_3 with which the gelatin was originally

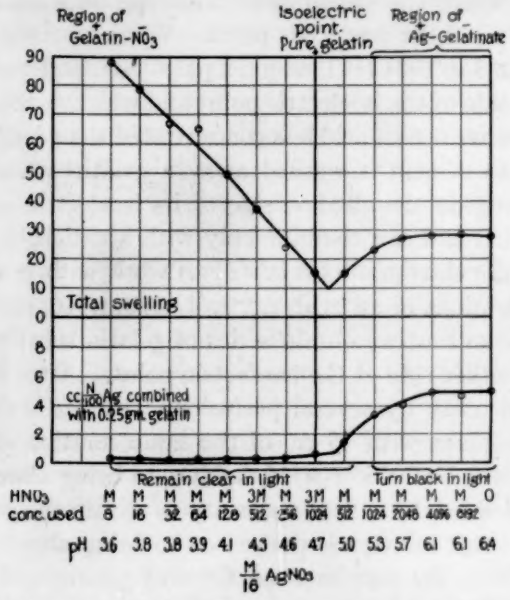

FIG. 1. Gelatin treated with different concentrations of HNO_3, from M/8 to M/8192, washed, and then treated with the same concentration of $AgNO_3$ (M/16), and then washed again. Abscissæ show concentrations of acid used. The final pH of the gelatin solution is found under the figure for the concentration of acid used.

The ordinates of the lower curve give the values for the silver found in combination with the gelatin. The curve shows that at the isoelectric point (pH = 4.7) and on the acid side of the isoelectric point, the gelatin was practically free from silver. On the more alkaline side the amount of silver found in combination with the gelatin increased with the pH. This proves that gelatin can combine with a cation only on the alkaline side from the isoelectric point, and this is corroborated by the fact that on the alkaline side from the isoelectric point only was the gelatin darkened by light. The ordinates of the upper curve are the values for the swelling of the same gelatin. On the alkaline side from the isoelectric point, where the gelatin had combined with silver, the curve for swelling runs parallel to the curve for silver gelatinate formed. It was, therefore, the relative mass of silver gelatinate formed which determined the physical properties of gelatin.

treated. Under each concentration is put the pH found for the gelatin solution at the end of the experiment. The ordinates of the lower curve are the numbers for the cc. of 0.01 N silver in combination with 0.25 gm. of gelatin. The curve shows that at the isoelectric point (pH = 4.7) the gelatin is free from ionic silver and that the same is true for all the gelatin on the acid side from the isoelectric point (pH 3.6 to 4.7). On the alkaline side from the isoelectric point the

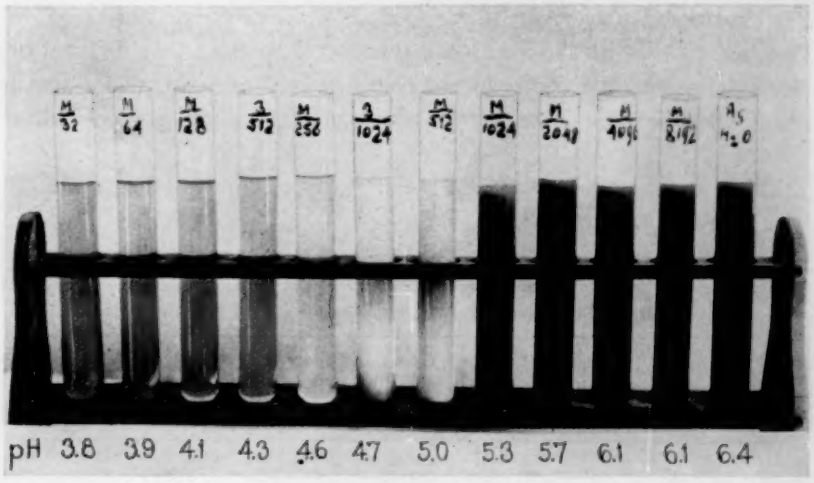

FIG. 2. Photograph of the gelatin solutions whose curves are contained in Fig. 1, taken a week after the experiment was made. The gelatin with a pH > 5.0 turned dark brown in a few hours when exposed to diffused daylight, while the gelatin with a pH ≦ 4.7 was not affected by light after an exposure of over a week. The photograph also shows that owing to the precipitate in suspension the tubes containing the gelatin at the isoelectric point, pH = 4.6 and 4.7, were more whitish than the rest, which were slightly yellow.

amount of ionic silver in combination with gelatin rises in a regular curve increasing in height with the increase in pH.

20 cc. of each gelatin solution were put into a test-tube and exposed to the light. The five test-tubes that had been treated with M/1024 or less concentrated HCl turned rapidly dark or black in the light, while none of the others did, even when made slightly alkaline and though the exposure lasted for more than a week. The gelatin treated

with 3 M/1024 acid (pH = 4.7) was quite opaque but remained perfectly white; while the test-tube treated with M/256 was only slightly opaque but remained also practically white. This shows that the gelatin treated with 3 M/1024 and whose pH was 4.7 was also entirely free from ionized silver. Fig. 2 is a photograph of these test-tubes. This experiment was repeated several times with the same result. It is a very striking demonstration experiment.

On the more acid side, where the gelatin was free from silver, the gelatin existed as gelatin nitrate as we shall prove presently. This then shows that at the isoelectric point and on the acid side from the isoelectric point a cation cannot combine with gelatin, while on the more alkaline side from the isoelectric point such a combination occurs.

Table I gives the cc. of 0.01 N Ag found in combination with 0.25 gm. of gelatin.

TABLE I.

Cc. 0.01 N Ag in combination with 0.25 gm. of gelatin at different pH.

pH	3.6	3.8	3.8	3.9	4.1	4.3	4.6	4.7	5.0	5.3	5.7	6.1	6.1	6.4
Cc. 0.01 N Ag combined with 0.25 gm. of gelatin	0.5	0.3	0.4	0.3	0.2	0.2	0.2	0.55	1.25	3.2	4.0	4.85	4.6	4.9

The small values of 0.2 cc. or even 0.5 cc. found in the gelatin on the acid side from the isoelectric point are due to incomplete removal by washing; an increase in the number of washings would probably have removed these traces also. Where 0.5 or less cc. of 0.01 N Ag was found in 0.25 gm. of gelatin, light no longer blackened the gelatin. As soon as pH became greater than 4.7 the value of Ag found rose suddenly.

Fig. 3 represents the physical properties of gelatin treated first with M/16 $AgNO_3$ and then brought to different hydrogen ion concentrations by treatment with different concentrations of HNO_3. 1 gm. of powdered gelatin was first put for 30 minutes into 100 cc. of M/16 $AgNO_3$ (pH about 6.9) and the excess of salt was then washed away (in the manner described). Then each gm. of gelatin was perfused three times with a definite concentration of HNO_3 and the excess of acid was washed away by four perfusions with distilled water. The

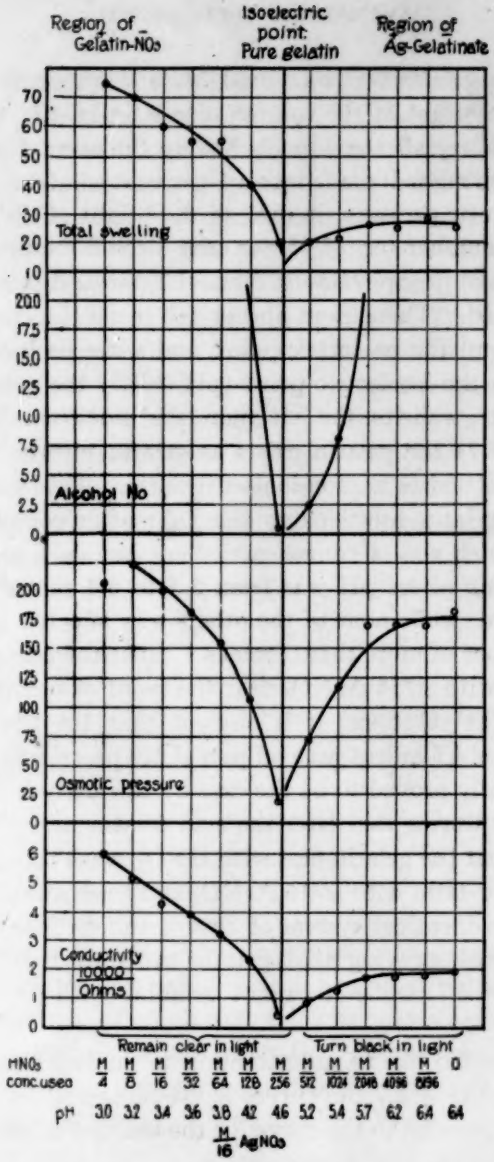
Region of Gelatin-NO3
Isoelectric point: Pure gelatin
Region of Ag-Gelatinate
Total swelling
Alcohol No
Osmotic pressure
Conductivity 10000/Ohms
Remain clear in light
Turn black in light
HNO3 conc. used
M/4 M/8 M/16 M/32 M/64 M/128 M/256 M/512 M/1024 M/2048 M/4096 M/8196 0
pH 3.0 3.2 3.4 3.6 3.8 4.2 4.6 5.2 5.4 5.7 6.2 6.4 6.4
M/16 AgNO3

FIG. 3. Gelatin treated first with M/16 $AgNO_3$, washed, then treated with different concentrations of HNO_3, and washed again. Curves for conductivity, osmotic pressure, alcohol number, and swelling. On the right from the isoelectric point the gelatin exists as silver gelatinate, on the left as gelatin nitrate, and at the isoelectric point as pure, sparingly soluble gelatin.

swelling of the gelatin was measured while it was in the cylindrical funnel and the height of the column of gelatin in mm. expresses the degree of swelling, all the funnels having the same diameter. The gelatin was then melted, made into a 1 per cent solution, and the conductivity, osmotic pressure (in mm. of the height of the gelatin solution), alcohol number (cc. of 95 per cent alcohol required to cause a definite degree of precipitation in 5 cc. of 1 per cent gelatin solution) were determined. There is an abrupt fall in all the curves near pH 4.6; *i.e.*, at about the isoelectric point, and a rise on both sides. On the right from the isoelectric point (pH > 4.7) the gelatin exists as silver gelatinate, while on the left, more acid side from the isoelectric point (pH < 4.7) the gelatin exists as gelatin nitrate. At the isoelectric point it exists as non-ionized gelatin. This was proved by exposing the gelatin solutions to the light after completion of the experiment which was of course carried out in a dark room; all those gelatin solutions whose pH was from 5.3 to 6.4 turned black when exposed to light, while none of the others was affected by light even after an exposure of more than 2 weeks. Since the original treatment of the gelatin with M/16 $AgNO_3$ near the point of neutrality was the same for all the solutions, it is obvious that the silver gelatinate when its pH was 4.7 or less gave off part if not practically all its silver. This was actually proved to be the case by the analysis of the filtrate of such gelatin during and after the acid treatment.

When we treat the gelatin first with HNO_3, wash the acid away, and then treat the gelatin with M/16 $AgNO_3$ and wash away the excess of salt, we get an identical system of curves and an identical effect of exposure to light, proving that gelatin can only combine with Ag when its pH > 4.7, while it cannot combine with gelatin when its pH $\leqq$ 4.7.

The writer would like to call attention to the fact shown in Figs. 1 and 3 that for pH > 4.7 the curves for swelling and for conductivity are practically parallel to the curve for the amount of silver gelatinate formed.

The Combination of Anions with Gelatin.

In this chapter we intend to furnish the data necessary for proving that gelatin combines with anions only when pH < 4.7, while it can-

not combine or remain in combination with anions when pH $\leqq$ 4.7. We chose for this purpose two anions, Br and CNS, for the volumetric determination of which we possess the convenient Volhard method. Powdered gelatin was first treated with different concentrations of HNO_3 and then washed free from the excess of acid. Then the gelatin in each funnel was perfused three times with 25 cc. of M/8 NaBr and this was followed by six perfusions with 25 cc. of H_2O. The swelling was plotted (upper curve in Fig. 4), the gelatin was melted and made into a 1 per cent solution, and analyzed for Br by the Volhard method. The lower curve gives the quantity of Br in combination with 25 cc. of gelatin. Table II gives the amount of Br found in combination with 0.25 gm. of gelatin. The table, as well as the curve, shows that the treatment of gelatin with M/8 NaBr did not lead to the formation of gelatin bromide when pH $\leqq$ 4.7; while it led to the formation of gelatin Br when pH < 4.7. The quantity of gelatin bromide formed increased with the hydrogen ion concentration.

TABLE II.

Cc. 0.01 N Br in combination with 0.25 gm. of gelatin at different pH.

pH	3.7	3.8	3.9	4.1	4.2	4.6	4.7	5.1	5.8	6.4	6.8	7.0	7.1
Cc. 0.01 N Br combined with 0.25 gm. of gelatin	7.5	7.4	7.1	5.0	2.95	1.4	0.1	0.15	0.1	0.15	0.2	0.1	0.1

In the experiment represented by Fig. 5 everything was the same except that the treatment of gelatin with HNO_3 was followed by three perfusions with M/8 NH_4CNS (instead of M/8 NaBr). The lower curve shows that when pH $\leqq$ 4.7 the gelatin contains no CNS, but that gelatin can combine with CNS as soon as pH < 4.7.

The results are given in Table III. The reader's attention is called to the sharp drop in the values for Br (Table II) as well as for CNS (Table III) at pH = 4.7 and less.

TABLE III.

Cc. 0.01 N CNS in combination with 0.25 gm. of gelatin at different pH.

pH	3.5	3.5	3.9	4.1	4.3	4.4	4.7	4.9	5.5	6.2	6.3	6.4	6.5	7.0
Cc. 0.01 N CNS combined with 0.25 gm. of gelatin	9.4	9.2	6.7	4.1	3.4	1.95	0.15	0.2	0.1	0.1	0.1	0.1	0.1	0

When we reverse the order of treatment, *i.e.* when we treat gelatin first with M/8 NaBr at pH about 7.0, only sodium gelatinate should be formed according to our theory, and no Br should combine with the gelatin. This was found to be correct. When we treat gelatin first

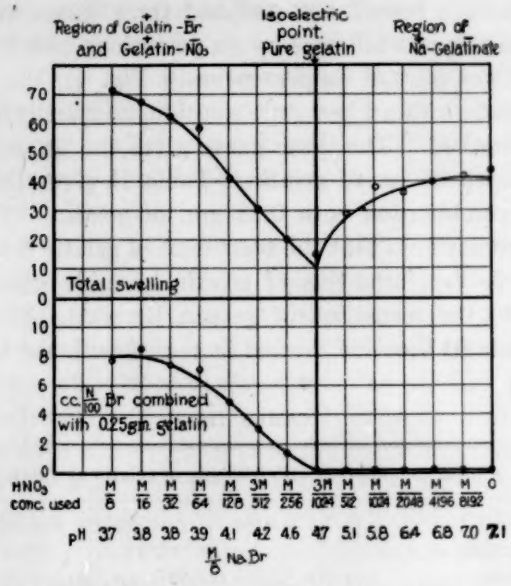

FIG. 4. Gelatin treated with different concentrations of HNO_3, washed, treated with M/8 NaBr, and washed again. The ordinates of the lower curve are the values of Br in combination with gelatin, showing that on the right (alkaline) side from the isoelectric point and at the isoelectric point gelatin contains no Br, while on the left, more acid side from the isoelectric point, the amount of the Br found increases with the pH. The ordinates of the upper curve represent the swelling of the same gelatin. The two curves on the left side are almost parallel, showing that the degree of swelling is determined by the relative mass of the gelatin bromide (or nitrate) formed.

with M/8 NaBr, then with varying concentrations of HNO_3, no Br should enter into ionogenic combination with the gelatin. This is confirmed by the volumetric analysis which shows that such gelatin is free from ionic Br. Only sodium gelatinate is formed under these conditions.

Figs. 4 and 5 confirm a fact already referred to in connection with Fig. 1. The curves for the swelling of gelatin for pH < 4.7 are parallel to the curves representing the amount of gelatin Br and gelatin CNS formed. Fig. 6 gives the relation of the curve for swelling to the curves for the other physical properties of gelatin treated with acid and then with M/8 NaBr. These experiments prove definitely that on the acid side from the isoelectric point gelatin (and probably amphoteric colloids in general) can combine only with anions, on the

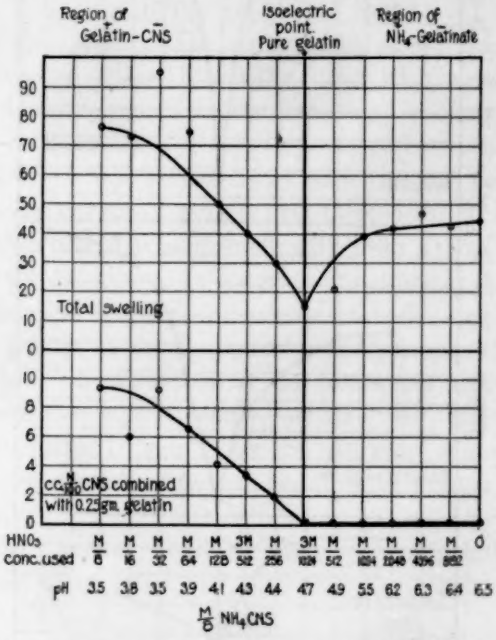

FIG. 5. Gelatin treated with different concentrations of HNO_3, washed, then treated with M/8 NH_4CNS, and washed again. Further explanation of figure corresponds to that for Fig. 4.

more alkaline side from the isoelectric point only with cations, and at the isoelectric point with neither.

Action of Heavy Metals and Polyvalent Ions.

When we treat finely powdered gelatin with a solution of M/10 copper acetate, the powder assumes a blue color due to the formation

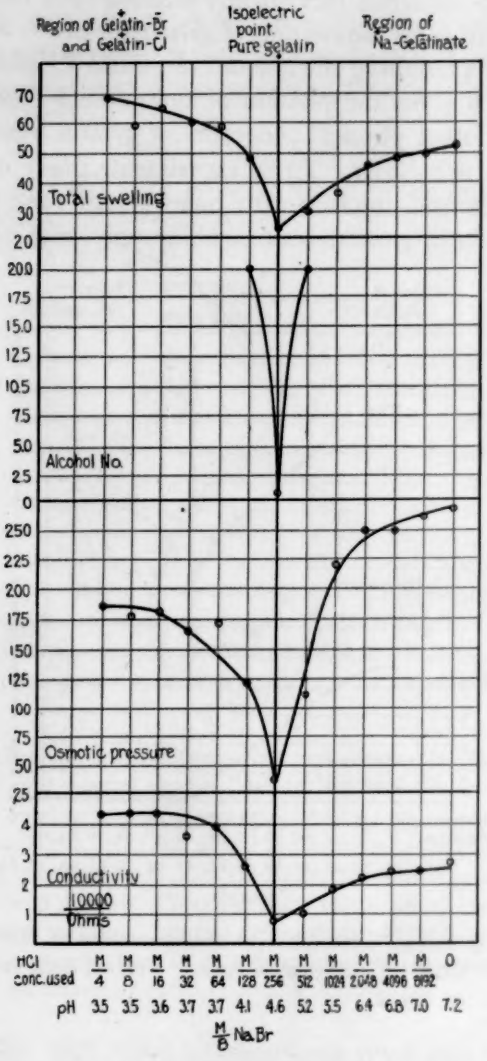
Region of Gelatin-Br and Gelatin-Cl
Isoelectric point. Pure gelatin
Region of Na-Gelatinate
Total swelling
Alcohol No.
Osmotic pressure
Conductivity 10000/Ohms
HCl conc. used
M/4 M/8 M/16 M/32 M/64 M/128 M/256 M/512 M/1024 M/2048 M/4096 M/8192 0
pH 3.5 3.5 3.6 3.7 3.7 4.1 4.6 5.2 5.5 6.4 6.8 7.0 7.2
M/8 NaBr

FIG. 6. Gelatin treated first with varying concentrations of HCl, washed, then treated with M/8 NaBr, and washed again. Curves for conductivity, osmotic pressure, alcohol number, and swelling. On the right side from the isoelectric point gelatin exists as sodium gelatinate, on the left side as gelatin chloride or bromide, at the isoelectric point as pure, sparingly soluble gelatin.

of copper gelatinate (pH being about 6.3). When we wash off the excess of copper acetate solution, the blue color remains. When we treat such copper gelatinate with an acid to increase the hydrogen ion concentration, we notice that the gelatin loses its blue color as soon as its pH $\leqq$ 4.7. If after the acid treatment gelatin is perfused six times with distilled water, the gelatin keeps its blue color when pH $>$ 4.7, and it is possible to demonstrate the presence of ionized copper in such gelatin with the ferrocyanide test. When the pH of the gelatin is $\leqq$ 4.7, the ferrocyanide test shows that the gelatin is free from ionized copper. The water with which this gelatin was washed contains the copper, while the water with which the gelatin with a pH $>$ 4.7 was washed is free from copper, except that which was originally contained in the capillary spaces between the granules of gelatin and which was carried away with the first perfusions.

Fig. 7 gives the curves of gelatin first treated for 30 minutes with M/16 copper acetate and subsequently with from M/8 to M/8192 HCl. For pH $\leqq$ 4.6 all the values of the curves are as low as they are at the isoelectric point, and for the same reason; namely, copper gelatinate as well as the gelatin at the isoelectric point being sparingly soluble. (It is well to keep in mind that at the isoelectric point the gelatin contains no ionizable copper, while for pH $>$ 4.7, the gelatin is in combination with ionic copper.) On the left, more acid side from the isoelectric point the gelatin exists in the form of gelatin chloride and gelatin acetate.

The experiment proves, therefore, that copper gelatinate cannot continue to exist when pH $\leqq$ 4.7, and that the gelatin gives off its copper at such pH.

The same experiment was made with $CeCl_3$ and Pb acetate$_4$, and almost identical curves were obtained as in the case of copper acetate, Ce as well as Pb forming sparingly soluble gelatinates. In the case of K_4FeCN_6 the sparingly soluble gelatin$_4$ $FeCN_6$ was formed only on the acid side of the isoelectric point as our theory demands. Fig. 8 gives the curves. The gelatin was first treated with different concentrations of HCl and then with M/16 K_4FeCN_6 in the manner described for the other cases.

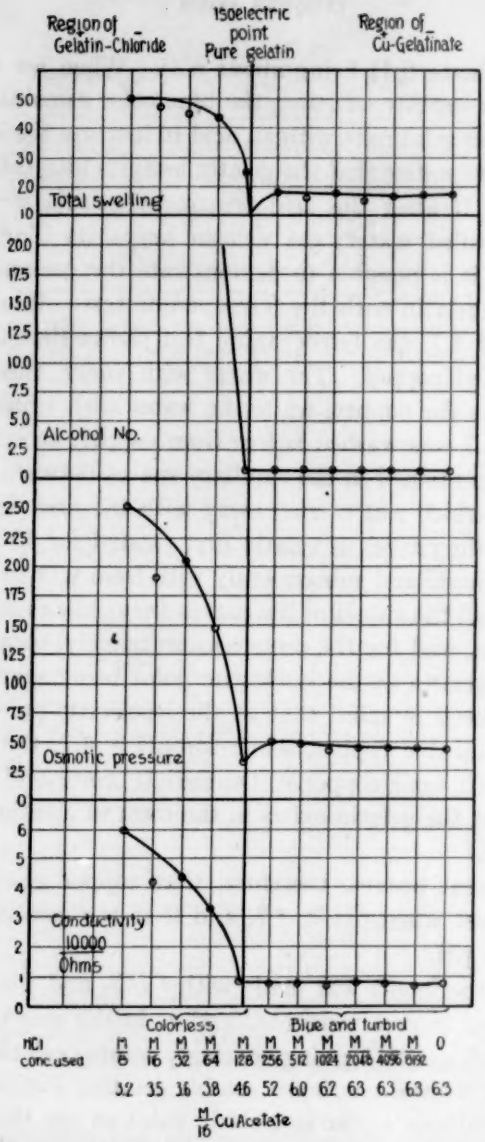
Region of + Gelatin-Chloride
Isoelectric point Pure gelatin
Region of + Cu-Gelatinate
Total swelling
Alcohol No.
Osmotic pressure
Conductivity 10000/Ohms
Colorless
Blue and turbid
HCl conc. used
M/8 M/16 M/32 M/64 M/128 M/256 M/512 M/1024 M/2048 M/4096 M/8192 0
3.2 3.5 3.6 3.8 4.6 5.2 6.0 6.2 6.3 6.3 6.3 6.3
M/16 Cu Acetate

FIG. 7. Gelatin treated with M/16 copper acetate, washed, treated with different concentrations of HCl, and washed again. On the right side from the isoelectric point gelatin exists as copper gelatinate, stained blue, practically insoluble, and with low conductivity, osmotic pressure, alcohol number, and swelling. On the left side from the isoelectric point it exists as gelatin chloride (and acetate), highly soluble, and with high conductivity, osmotic pressure, alcohol number, and swelling; at the isoelectric point pure gelatin, practically insoluble.

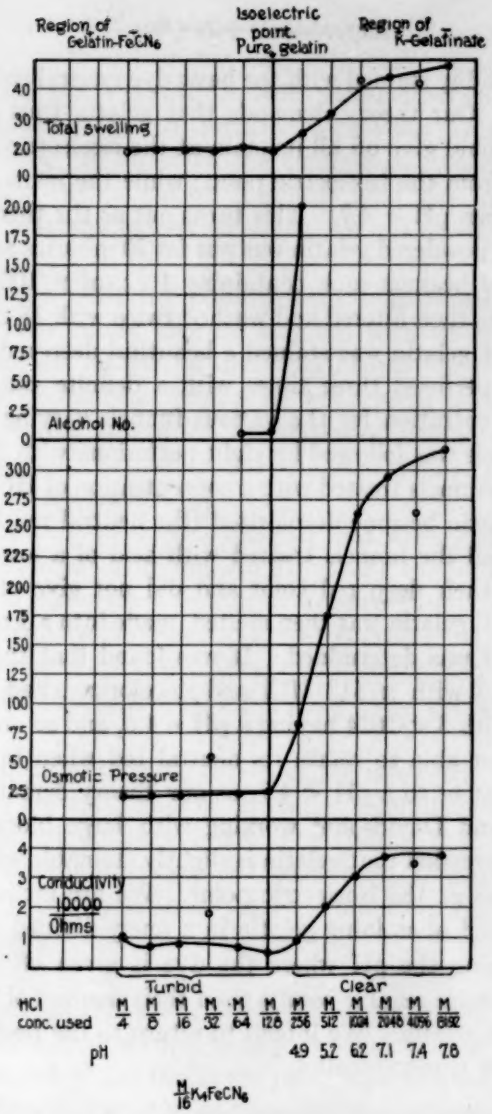

FIG. 8. Gelatin treated with different concentrations of HCl, washed, then treated with M/16 K_4FeCN_6, and then washed again. On the left, more acid side from the isoelectric point, gelatin exists as insoluble gelatin ferrocyanide, with low osmotic pressure, low conductivity, etc.; on the right, more alkaline side from the isoelectric point, gelatin exists as potassium gelatinate with high conductivity, osmotic pressure, etc.; at the isoelectric point, pure and sparingly soluble gelatin.

Experiments with Dyes.

Gelatin is readily stained with the basic dye neutral red at the point of neutrality. Our theory demands that gelatin thus stained with neutral red should give off all its stain at the isoelectric point and on the acid side from the isoelectric point; while the neutral red should be retained when pH > 4.7. This turns out as the theory demands. 1 gm. of finely powdered gelatin was put for 20 minutes at about 15°C. into a series of beakers each containing 1 cc. of M/100 neutral red. The gelatin was then filtered and washed twice with 25 cc. of distilled water. All the gelatin was stained a beautiful deep red. Each funnel was then perfused three times with a definite concentration of HCl, the concentration for the various funnels varying from M/8 to M/8192, and this was followed by eight perfusions with 25 cc. of H_2O each. In the funnels treated with a concentration of HCl of M/512 or above, the gelatin became decolorized (the neutral red being washed away); while all the funnels treated with acid of a lower concentration retained their deep red color and did not give off their stain. The decolorized gelatin was then melted, made into a 1 per cent solution whose pH was determined. It was found that the gelatin previously treated with M/512 HCl and decolorized had a pH = 4.7, that treated with 3 M/1024 having a pH = 4.6, and so on. Hence the gelatin was not able to retain its neutral red when brought to the isoelectric point or to a pH $\leqq$ 4.7, as our theory demands.

Michaelis and Davidsohn[3] working with large blocks of gelatin, gained the impression that gelatin is slightly stainable with both basic and acid stains at the isoelectric point. We are inclined to believe that our method of staining gelatin in a finely divided condition and then ascertaining the pH where the dye is given off again by the gelatin, gives more reliable results than their method of working with large blocks of gelatin. We intend to return to the problem of staining in a future communication.

SUMMARY.

1. It is shown by volumetric analysis that on the alkaline side from its isoelectric point gelatin combines with cations only, but not

[3] Michaelis. L., and Davidsohn, H., *Biochem. Z.*, 1913, liv, 323.

with anions; that on the more acid side from its isoelectric point it combines only with anions but not with cations; and that at the isoelectric point, pH = 4.7, it combines with neither anion nor cation. This confirms our statement made in a previous paper that gelatin can exist only as an anion on the alkaline side from its isoelectric point and only as a cation on the more acid side of its isoelectric point, and practically as neither anion nor cation at the isoelectric point.

2. Since at the isoelectric point gelatin (and probably amphoteric colloids generally) must give off any ion with which it was combined, the simplest method of obtaining amphoteric colloids approximately free from ionogenic impurities would seem to consist in bringing them to the hydrogen ion concentration characteristic of their isoelectric point (*i.e.*, at which they migrate neither to the cathode nor anode of an electric field).

3. It is shown by volumetric analysis that when gelatin is in combination with a monovalent ion (Ag, Br, CNS), the curve representing the amount of ion-gelatin formed is approximately parallel to the curve for swelling, osmotic pressure, and viscosity. This fact proves that the influence of ions upon these properties is determined by the chemical or stoichiometrical and not by the "colloidal" condition of gelatin.

4. The sharp drop of these curves at the isoelectric point finds its explanation in an equal drop of the water solubility of pure gelatin, which is proved by the formation of a precipitate. It is not yet possible to state whether this drop of the solubility is merely due to lack of ionization of the gelatin or also to the formation of an insoluble tautomeric or polymeric compound of gelatin at the isoelectric point.

5. On account of this sudden drop slight changes in the hydrogen ion concentration have a considerably greater chemical and physical effect in the region of the isoelectric point than at some distance from this point. This fact may be of biological significance since a number of amphoteric colloids in the body seem to have their isoelectric point inside the range of the normal variation of the hydrogen ion concentration of blood, lymph, or cell sap.

6. Our experiments show that while a slight change in the hydrogen

ion concentration increases the water solubility of gelatin near the isoelectric point, no increase in the solubility can be produced by treating gelatin at the isoelectric point with any other kind of monovalent or polyvalent ion; a fact apparently not in harmony with the adsorption theory of colloids, but in harmony with a chemical conception of proteins.

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